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THE POTENTIATING ACTION OF ACETYLCHOLINE
ON THAT OF ADRENALINE

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In the course of experiments on the stimulating action of acetylcholine on the heart (McDowall, 1946) it was observed that acetylcholine also sensitizes the organ to the action of a subsequent injection of adrenaline. The present investigation was carried out to determine how far the sensitizing action might be a general phenomenon. It was found that acetylcholine sensitizes the heart, the vessels, the blood pressure, the pupil and the intestine to the action of adrenaline.

RESULTS

Effect on the heart. Hearts of rabbits and cats were perfused with Ringer-Locke's solution by the usual method (McDowall, 1946). Fig. 1 shows the potentiating effect of injecting acetylcholine on the response to adrenaline on the cat's heart. The result was the same for the rabbit's heart. In this, and in all subsequent experiments, adrenaline hydrochloride (Parke, Davis and Co.) was injected. It must be emphasized that a demonstration of the sensitizing effect of acetylcholine on the heart, as well as on other organs, cannot readily be repeated on the same preparation, as the increased sensitivity is often prolonged.

Effect on the blood-vessels. The method used was that described by Hemingway & McDowall (1926) in which the hind legs of a cat are perfused with Ringer's solution under a constant pressure, a record being taken of the change in the resistance to the flow, measured by a side-tube from the cannula entering the femoral artery. The drugs are injected through the rubber tubing leading to the cannula.

A dose of adrenaline, sufficient to cause a moderate constriction of the vessels, is injected and after the constriction has passed off, the vessels are dilated by means of acetylcholine (Fig. 2). Fig. 2 also shows the subsequent response to adrenaline; although, after the acetylcholine, the resistance is below that originally present, the rise caused by adrenaline is increased.

The sensitizing action on blood-vessels may also be seen if the volume of the venous outflow from the limbs is recorded in a chloralosed cat, in which coagulation of the blood is prevented by the injection of chlorazol fast pink.
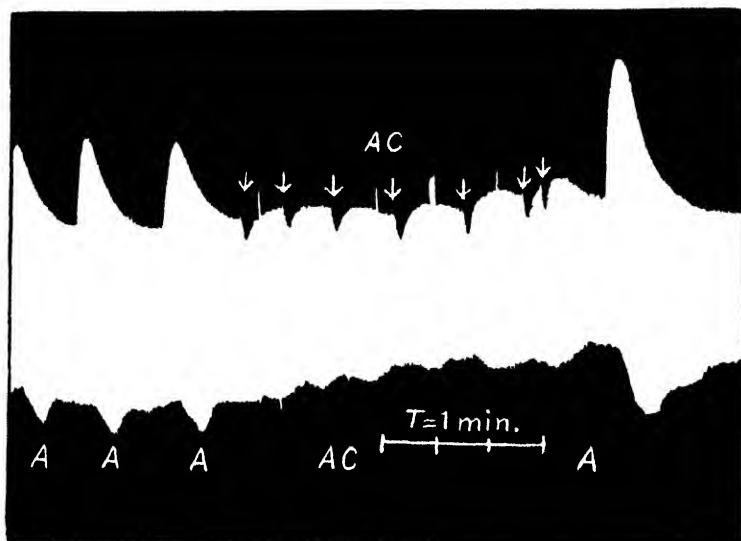
Fig. 1. Perfused cat's heart. Effect of seven injections of 0·5 μg. acetylcholine (at arrows) in increasing the action of 0·5 μg. adrenaline (at *A*). Time in min.
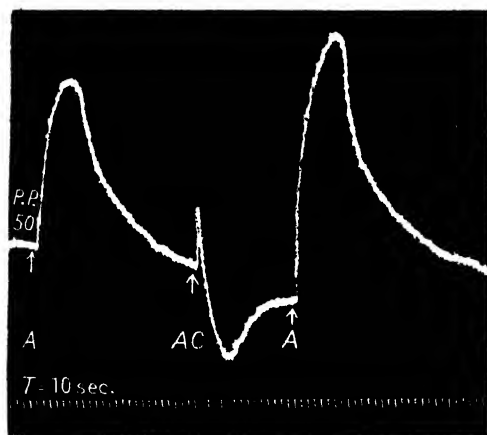
Fig. 2. Perfusion of blood-vessels of a cat's hind-limb. Record of perfusion pressure (P.P.). Effect of 0·5 μg. of acetylcholine (at *AC*) on the constrictor response to 0·25 μg. adrenaline (at *A*), P.P. = perfusion pressure, mm. H_2O. Time in 10 sec.

This sensitizing action on blood-vessels has already been reported by Danielopolu & Marcu (1939).

Effect on the blood pressure. This was studied on cats under chloralose anaesthesia. If the effects on the arterial blood pressure of intravenous injection of adrenaline before and after acetylcholine are compared, the results are varied. There may be an increased effect, no alteration or a reduced effect. The cause of the variation becomes apparent if simultaneous plethysmograph records are taken of corresponding skinned and unskinned limbs. The acetylcholine enhances both the vasodilator and vasoconstrictor effect of adrenaline, the former being best seen on the skinned limbs, where vessels of muscles predominate, and the latter in the normal limb during the first few minutes after the injection of acetylcholine, when the effect on the skin vessels predominates.
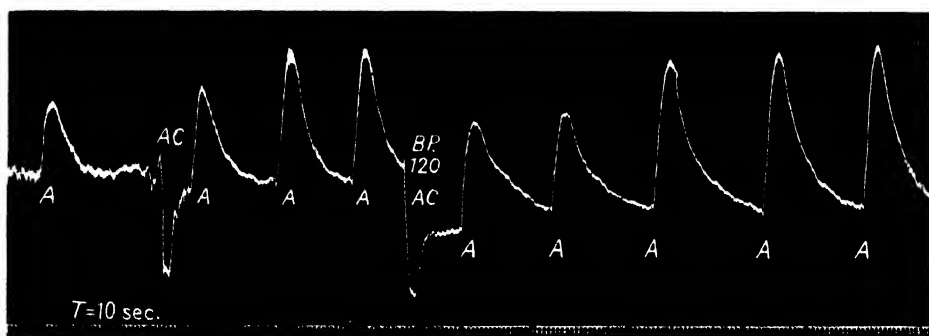
Fig. 3. Record of arterial blood pressure of a cat under chloralose. Effect of intravenous injection of $10\,\mu g$. acetylcholine (at *AC*) on response to intravenous injection of $5\,\mu g$. adrenaline (at *A*). Time in 10 sec.

In order to demonstrate the potentiating action of acetylcholine on the vasoconstrictor response, it is essential that the sympathetic, which is initially stimulated by chloralose (Vincent & Thompson, 1928) should be at rest. This state is reached by keeping the anaesthetized animal warm and undisturbed for about an hour, and is recognized by the fact that the pupils are constricted, though they still react to light and to sensory stimulation (McDowall, 1925). The record shown in Fig. 3 was obtained at this stage. At later stages in such experiments there is commonly a sustained constriction of the skin vessels, and in this condition the vasodilatation of muscle vessels by adrenaline predominates; this is now seen to be enhanced by the acetylcholine, with the result that the response of the blood pressure is reduced (Fig. 4). It may be noted that the dilatation of the vessels of the muscles is not a function of the rise of blood pressure.

Effect on the pupil. If a dose of about $0.5\,\mu g$. of adrenaline is injected intravenously into a chloralosed cat, rested as above, the pupil is dilated very slightly. In this case, however, since the action of acetylcholine is so short-lived, owing to its rapid destruction in the blood, the sensitizing effect of

acetylcholine is not seen unless eserine is also injected at the same time to prevent the destruction. Subsequently, a similar dose of adrenaline may cause a full dilatation of the pupil.

Effect on the isolated intestine. The inhibitory action of adrenaline on the tone and movements of a piece of duodenum suspended in warm oxygenated Tyrode's solution is well known, and, with small doses, the effect is usually quite short-lived (Jendrassik, 1924). Generally, a repetition of the application of adrenaline to a fresh preparation results in a reduction of the response.
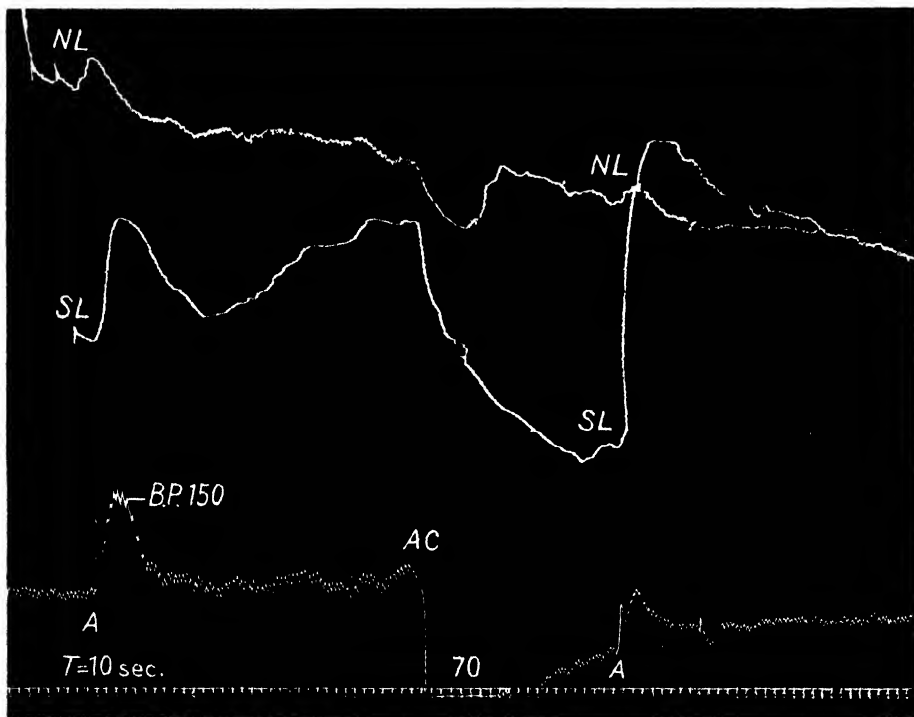
Fig. 4. Cat under chloralose. Effect of intravenous injection of 50 μg. of acetylcholine (at *AC*) on the response to an intravenous injection of 5 μg. adrenaline (at *A*). Upper record (N.L.) volume of normal limb in plethysmograph. Middle record (S.L.), volume of skinned limb in plethysmograph. Foot tied off. Lower record, arterial blood pressure. Time in 10 sec.

If a dose of adrenaline is used which is just sufficient to depress the intestine (the actual amount varies in different preparations) it is observed that if, after washing with fresh Tyrode's solution, a dose of acetylcholine is added to the bath, and the preparation again washed with fresh solution, the addition of a subsequent dose of adrenaline is often followed by an enhanced response. This may show itself either in a greater reduction of tone or in a more prolonged depression of the intestinal movements (Fig. 5). In some cases, however, the opposite result was obtained, and in spite of some fifty attempts, it has not been possible to determine the exact cause of this inconsistency.

More consistent results are obtained if a preparation is used which has been desensitized to adrenaline by several recent doses of adrenaline itself. If to such a preparation acetylcholine is added and washed off, the full sensitivity returns, and indeed the adrenaline may be more effective than originally (Fig. 6). Numerous attempts were made to demonstrate the sensitizing effect of the acetylcholine after atropine, but were unsuccessful because of the well-known variable effects of atropine itself on the intestine. In some cases atropine inhibits the intestine completely, and in others it appears to be ineffective.
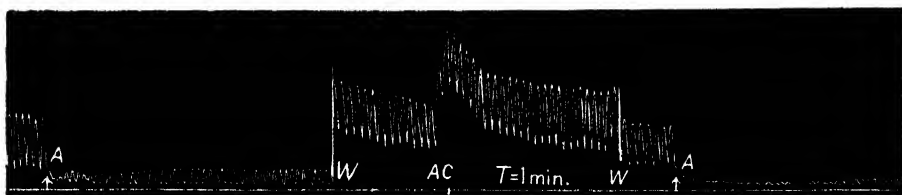
Fig. 5. Contractions of longitudinal muscle of isolated duodenum of rabbit suspended in Tyrode's solution. Potentiating effect of 13 μg. of acetylcholine (at *AC*) on the response to 6·5 μg. of adrenaline (at *A*). At *W* the bath was washed out and refilled with fresh Tyrode's solution.
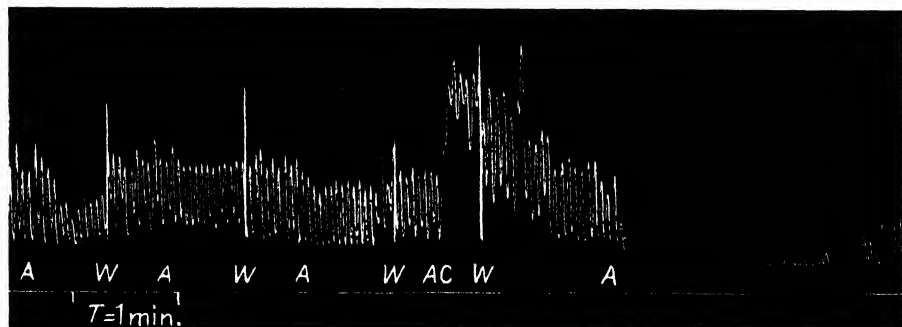
Fig. 6. Contractions of longitudinal muscle of isolated duodenum of rabbit suspended in Tyrode's solution. Sensitizing effect of 0·1 μg. of acetylcholine (at *AC*) on response to 1 μg. of adrenaline (at *A*). Muscle rendered insensitive to 0·1 μg. adrenaline by repeated application. At *W* the bath was washed out and refilled with fresh Tyrode's solution.

DISCUSSION

Sensitization of tissues by acetylcholine to the action of adrenaline is widespread, and may be shown on the heart, blood-vessels, arterial blood pressure, pupil and intestine. The exact nature of the sensitization is not clear. If it is agreed that the acetylcholine stimulates chromaffin tissue which may liberate adrenaline, as suggested by Hoffmann, Hoffmann, Middleton & Talesnik (1945) in relation to the heart, then it must be assumed that such chromaffin tissue is much more widespread than is generally recognized. It is equally possible, however, that the sensitization is some direct effect on the contractile elements

of the heart. It is unlikely that the sensitization is due to stimulation of sympathetic ganglia, since the effect occurs also on the perfused vessels of the hind limbs, in which no ganglia have been described. The results generally are in accordance with a number of observations made by various workers on other parts of the body.

A synergic action of adrenaline and acetylcholine has already been described by Dale & Gaddum (1930) in relation to denervated muscle, by Bülbring (1945) in relation to the stimulation of the rectus abdominis of the frog, and by Bülbring & Burn (1942) in relation to transmission at the nerve-endings of a nerve-muscle preparation, while many clinical observers have emphasized the beneficial action of ephedrine in myasthenia gravis. A similar action of adrenaline and acetylcholine on the urinary bladder has been described by Mellanby & Pratt (1939, 1940). Such sensitization may have a considerable functional significance in relation to the parasympathetic system because adrenaline is secreted under conditions of emotional or physical stress at a time when parasympathetic action (i.e. the action of the vagus) is correspondingly reduced. The secretion of acetylcholine, however, just prior to the reduction may be looked upon as being partly responsible for the immediate increase of cardiac action, dilatation of the pupil and inhibition of the intestine produced by adrenaline. Thus the value of continuous liberation of acetylcholine by the parasympathetic system, which hitherto has appeared to be largely an extravagance of nature, becomes apparent.

The effect on the intestine is of special interest, as it suggests a new function for the abdominal vagus. It has always been difficult to understand why the action of adrenaline on the isolated intestine is so variable and often of such a short duration, while that in vivo is prolonged. In the isolated intestine the vagus has been severed and while, in some animals, there may remain some activity originating in the intestinal parasympathetic ganglia, as indicated by the action of atropine, such activity is not constant. This variability may merely reflect that of the vagus generally, which is known to be related to habitual activity. Thus it may be considered that animals of more active habit, which produce large amounts of acetylcholine at the vagus endings, are sensitized thereby to the action of adrenaline, and are thus made more efficient for exercise.

SUMMARY

Acetylcholine increases the response of the heart, blood-vessels, arterial blood pressure, pupil and intestine to adrenaline. The possible significance of the effect in relation to parasympathetic activity is discussed.

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THE EFFECTS OF MAGNESIUM IONS AND OF CREATINE PHOSPHATE ON THE SYNTHESIS OF ACETYLCHOLINE

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This paper deals with the influence of divalent cations on the synthesis of acetylcholine and with the problem of determining whether creatine phosphate (CrP) can replace adenosine triphosphate (ATP) in this synthesis.

In previous experiments in which studies were made of the synthesis of acetylcholine by homogenized brain tissue (Nachmansohn & Machado, 1943) or by acetone-dried brain (Feldberg & Mann, 1945) in the presence of ATP, it was found that magnesium ions had little or no action. In later experiments (Feldberg & Hebb, 1945), in which *dialysed* enzyme preparations of acetone-dried brain tissue were used, Mg ions were found to have a strong accelerating action on the synthesis of acetylcholine; it appeared as though dialysis had removed from the system the Mg ions, in the absence of which synthesis was greatly depressed. This explanation is only partially true. Dialysis is not the sole determining factor. A distinction has to be made between the synthesis which is accelerated by citrate and the synthesis which occurs in its absence. It has now been found that only the synthesis accelerated by citrate is potentiated by Mg ions, although, in order to obtain the maximum effect, dialysed enzyme preparations have to be used. We have further shown that Mg ions can be replaced by manganese ions, but not by any of the other divalent cations examined.

The suggestion has been made (v. Muralt, 1943; Nachmansohn, Cox, Coates & Machado, 1943) that not only ATP but also CrP may act in the synthesis of acetylcholine in a manner analogous to their action in muscle metabolism. We have, in fact, found that, under certain conditions, CrP can replace ATP in the synthesis of acetylcholine. A short account of these results has been published (Feldberg & Hebb, 1946). Since then Torda & Wolff (1946), using minced frog's brain as the source of their enzyme preparations, have found

independently that CrP increases the formation of acetylcholine by about 50% and that creatine produces about half this effect. With our method the accelerating effect of CrP is incomparably greater, but creatine is inactive.

METHODS

The experimental methods were essentially the same as those previously described by Feldberg & Mann (1945, 1946). Non-dialysed or dialysed saline extracts were prepared from the acetone-dried brain of the rat or the guinea-pig, and incubated aerobically. In a few experiments incubation was carried out anaerobically. The incubated samples always had a volume of 4·5 c.c. and included, unless otherwise stated, the following: saline extract of 50 mg. acetone-dried brain powder, 6 mg. KCl, 3 mg. choline, 2 mg. NaF, 4·5 mg. cysteine, 0·5 mg. eserine sulphate and either 1·5 mg. MgSO$_4$ or 2–4 mg. MgCl$_2$. In testing the effects of other divalent cations, the salts used were CaCl$_2$, MnCl$_2$, ZnSO$_4$ and Co(NO$_3$)$_2$. Activator when required was prepared by boiling saline extracts of acetone-dried brain tissue; the supernatant fluid of the boiled extract, after centrifugation, was added to the sample in an amount equivalent to 100 mg. acetone powder Citrate when used was added in an amount of 15 mg. sodium citrate; similarly, the amount of ATP per sample, when it was used, was 0·4 mg. ATP-pyro P. About 5 mg. of the barium salt (converted to the sodium salt before use) correspond to 1 mg. ATP-pyro P. CrP was kindly prepared for us as the Ba salt by Dr P. Eggleton and Dr R. N. Smith, to whom we should like to express our thanks. The barium salt was also converted to the sodium salt before use, and the amounts given in each sample are expressed in mg. creatine phosphate phosphorus (CrP-P), about 12 mg. of the barium salt correspond to 1 mg. Cr P-P. We should like also to express our thanks to Dr Malcolm Dixon for supplying us with a sample of muscle adenylic acid and to Dr S. Bach for supplying us with a sample of l(+)-glutamic acid.

RESULTS

Divalent cations

Magnesium ions. When synthesis of acetylcholine is produced in saline extracts of acetone-dried brain in the presence of ATP, KCl, NaF, choline, cysteine and eserine, Mg ions have no effect. If, however, citrate is added to the synthesizing medium as well, Mg ions exert a relatively strong accelerating action on the formation of acetylcholine. This difference is shown in the experiments of Table 1. In none of the nine experiments in which the synthesis was studied in the absence of citrate did Mg^{++} accelerate the synthesis, but in all but one of the fifteen experiments in which it was studied in the presence of citrate Mg ions had a strong action of this kind. This action was not affected by fluoride; it could be demonstrated in the absence of cysteine under anaerobic conditions (Table 1).

In Fig. 1 is seen the effect of varying concentrations of Mg ions on the synthesis of acetylcholine in the presence of citrate. A concentration of 0·0001 M increases the synthesis by about 40%; the optimal effect, an increase of about 300%, was obtained with a concentration of 0·004 M. With higher concentrations of Mg ions the rate of synthesis declined.

The action of Mg ions on the synthesis of acetylcholine in the presence of citrate was even more pronounced when the saline extracts used for incubation

TABLE 1. Effect of Mg ions on the formation of acetylcholine in non-dialysed saline **extracts of**
acetone-dried brain in the absence and in the presence of citrate

μg. acetylcholine formed in 1 hr./g. acetone powder

	Without citrate		With citrate		
Exp.	(a) No Mg	(b) 0·002 M-Mg	(c) No Mg	(d) 0·002 M-Mg	Notes
1	80	85	280	650	Anaerobically without cysteine
2	140	155	—	—	—
3	180	165	360	1050	—
4	180	190	430	1050	—
5	260	250	400	1100	—
6	450	330	720	1240	Activator added to samples
7	470	400	800	1100	Activator added to samples
8	180	190	330	700	Ca (0·002 M) added to samples
9	450	450	600	880	Activator and Ca (0·002 M) **added** to samples
10	—	—	140	420	NaF omitted from samples
11	—	—	310	880	—
12	—	—	460	510	NaF omitted from samples
13	—	—	520	880	NaF omitted from samples
14	—	—	560	900	—
15	—	—	610	1260	NaF omitted from samples
16		—	430	1250*	—

* Sample contained 0·004 M-Mg.
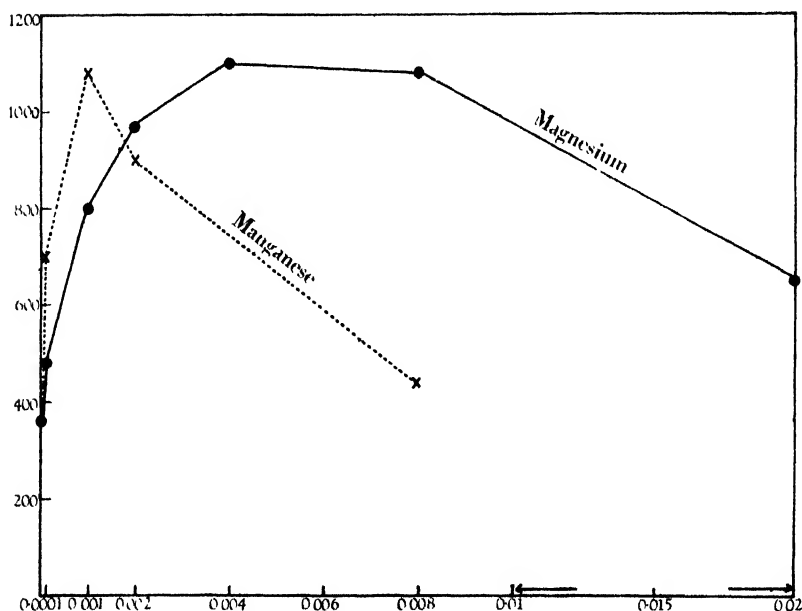
Fig. 1. Effect of varying concentrations of Mg ions (continuous line) and of Mn ions (dotted line) on the formation of acetylcholine by *non-dialysed* saline extracts of acetone-dried brain in the presence of ATP and citrate. Abscissae: molar concentration of Mg and Mn ions. Ordinates: μg./g./hr. acetylcholine formed.

were first dialysed. Again, fluoride did not affect the result. In such dialysed extracts Mg ions may increase the synthesis of acetylcholine by as much as 20 times (Table 2). In fact, the strong reactivating effect that citrate has on the synthesis in dialysed brain extracts in the presence of ATP depends on the Mg ions. For instance, in most experiments of Table 2, citrate increased the synthesis of acetylcholine by not more than 2 to 3 times in the absence, but 10–20 times in the presence of Mg ions. From Exps. 1 and 2 of Table 2 it will be seen that in dialysed extracts Mg ions have some accelerating effect on the synthesis of acetylcholine independent of citrate; the effect, however, is small compared to that obtained in the presence of citrate.

TABLE 2. Effect of Mg and Mn ions on the formation of acetylcholine in *dialysed* saline extracts of acetone dried brain in the absence and in the presence of citrate

μg. acetylcholine formed in 1 hr./g. acetone powder

	Without citrate		With citrate		
Exp.	No Mg	0·002–0·004 M-Mg	No Mg	0·002–0 004 M-Mg	0·001–0 002 M-Mn
1	12	28	24	440	—
2	18	35	55	580	—
3	20	—	30	600	—
4	12	—	38	380	500
5	12	—	55	500	620
6	20	.	25	330	420
7	24	—	64	430	520
8	40	—	150	650	720

In Fig. 2 is seen the effect of varying concentrations of Mg ions on the synthesis of acetylcholine in dialysed extracts and in the presence of citrate. Without Mg ions 24 μg. acetylcholine per g. tissue were formed in 1 hr.; a concentration of 0·00005 M-Mg ions, however, increased this value to 42, and a concentration of 0·002–0·004 M was usually necessary to obtain the maximal value which, in this experiment, was about 400 μg. With a concentration of Mg ions stronger than 0·004 M the synthesis of acetylcholine showed no further improvement, but was in fact slightly depressed.

The action of Mg ions when tested in dialysed saline extracts to which activator had been added is indistinguishable from its action on the synthesis in non-dialysed extracts, i.e. in the presence of citrate 0·002 M-Mg ions increased the synthesis about 2–3 times; in the absence of citrate it had no effect, or might even depress it. This was the case when large amounts of activator were added to the dialysed as well as to the non-dialysed enzyme extracts (see Exps. 6 and 7, Table 1).

Calcium ions. Ca ions are known to inhibit the synthesis of acetylcholine. According to Greville & Lehmann (1944) and to Bailey & Webb (1944), Ca ions and Mg ions have an antagonistic action on certain enzymes which is due to their being competitive with one another. This explanation does not account

for the opposing actions of Ca and Mg ions on the synthesis of acetylcholine. The accelerating action of Mg ions is dependent on citrate; the inhibiting action of Ca ions is not; in addition, Mg ions exert the accelerating effect in the presence of calcium ions (see Exps. 8 and 9, Table 1).

Manganese ions. Mn ions can replace Mg ions in the synthesis of acetylcholine. Like Mg ions the action of Mn ions is dependent on the presence of citrate. Of the two ions, Mn is the more powerful. In the experiment shown in Fig. 1, for instance, 0·001 M-Mn ions had the same optimal accelerating action on the synthesis of acetylcholine as Mg ions in a concentration of 0·004 M. Similarly, the depression of the synthesis which occurs with higher
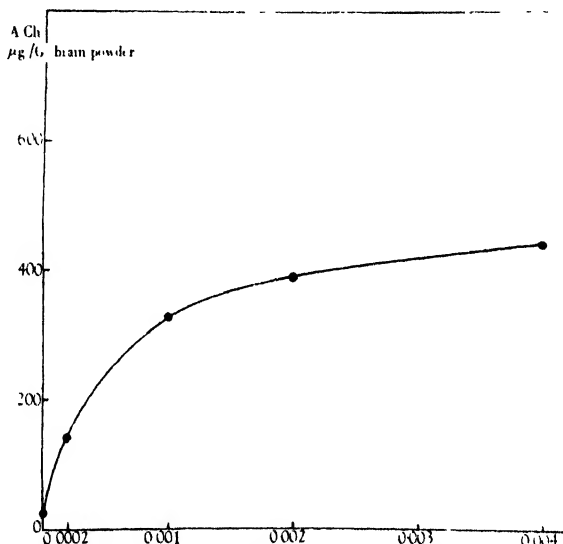
Fig. 2. Effect of varying concentrations of Mg ions on the formation of acetylcholine by *dialysed* saline extracts of acetone-dried brain in the presence of ATP and citrate. Abscissae: molar concentration of Mg ions. Ordinates: μg./g./hr. acetylcholine formed.

concentrations of Mg ions begins at 0·002 M-Mn ions. For the formation of acetylcholine in dialysed enzyme extracts also, the optimal concentration of Mn^{++} is lower than that of Mg^{++}. In the five experiments of Table 2, in which the effect of Mn and Mg ions were compared with each other, the amounts of acetylcholine formed in the presence of Mn were each time more than those formed in the presence of Mg^{++}, although the concentration of Mg^{++} was twice that of Mn^{++}.

By combining the two ions, Mg and Mn, in half-optimal concentrations, the synthesis of acetylcholine may be increased to values corresponding to those obtained with the optimal concentration of one ion alone. It is not possible, however, to increase the synthesis any further by combining the two ions each in optimal concentration. In fact, under such conditions, the synthesis of

acetylcholine decreases again, as it would do if one of the two ions alone were used in double the optimal concentration. From these experiments, which were carried out with dialysed enzyme extracts, it appears that the two ions Mg and Mn are interchangeable in their mode of action on the synthesis of acetylcholine.

Cobalt and zinc ions. These ions in a concentration of 0·0005–0·0001 M have no action on the synthesis of acetylcholine in dialysed or non-dialysed enzyme extracts, either in the presence or absence of citrate. Actually 0·004 M-Zn^{++} inhibited the synthesis.

Creatine phosphate (CrP)

Non-dialysed extracts. CrP may be used to replace ATP in the synthesis of acetylcholine by non-dialysed extracts prepared from acetone-dried brain powder. Of the two, CrP usually has the more powerful action. The maximal effect of CrP is obtained when it is used in a concentration of about 0·1 mg./c.c. CrP-P; some action may be observed even with a fiftieth of this concentration. The effects of this range of concentrations of CrP are seen in Table 3. For each

TABLE 3. Effect of different concentrations of CrP on the formation of acetylcholine in saline extracts of acetone-dried brain

Concentration of CrP-P in mg./c.c.	μg. acetylcholine formed in 1 hr./g acetone-powder	
	Without citrate	With citrate
0	23	45
0·002	45	75
0·01	170	230
0·05	250	900
0·1	300	1100

of the given concentrations of CrP, values are given for the amounts of acetylcholine synthesized both in the presence and absence of citrate, Mg ions being added in both cases. The synthesis of acetylcholine was much greater in the presence of citrate, but the effect of citrate depended largely on the addition of Mg ions as well. In this and other ways the action of CrP is analogous to that of ATP. For example, when CrP is used to replace ATP, the synthesis occurs also in the absence of fluoride and under anaerobic conditions; it is slightly increased by l(+)-glutamic and inhibited by pyruvic acid.

When one of the two, CrP or ATP, is already present in about optimal concentration, the synthesis is not very much increased by addition of the other. In the experiment of Table 4 are shown the effects of CrP and of ATP added either separately or together. A concentration of 0·08 mg./c.c. CrP-P brought the synthesis of acetylcholine to the value of 290 μg./g. in the absence of citrate and to 1200 μg./g. in the presence of citrate. With 0·09 mg./c.c. ATP-P the corresponding values were 145 and 900, and with 0·009 mg./c.c.

ATP-P 50 and 270 μg./g./hr. respectively. When the smaller concentration of ATP was given together with the CrP, the synthesis was increased by only 3%: when the stronger concentration was given together with CrP, by only 17% above the level of CrP alone.

TABLE 4. Effect of ATP and CrP on the formation of acetylcholine in saline extracts of acetone-dried brain

	μg. acetylcholine formed in 1 hr./g. acetone powder	
Additions of CrP-P or ATP-P in mg./c.c.	Without citrate	With citrate
None	26	80
0·009 ATP-P	50	270
0·09 ATP-P	145	900
0·08 CrP-P	290	1200
0·08 Cr-P, 0·009 ATP-P	300	1240
0·08 Cr-P, 0·09 ATP-P	340	1400

Dialysed extracts. On dialysis, the saline extracts of acetone-dried brain lose their ability to synthesize acetylcholine or retain this ability to only a slight extent, even in the presence of ATP. They are reactivated, however, by adding either ATP together with activator, or ATP together with citrate and Mg ions (Feldberg & Mann, 1946). In corresponding experiments with CrP, it was found that CrP together with activator reactivated the dialysed extract. CrP together with citrate and Mg ions on the other hand had no such effect. If, however, muscle adenylic acid were added as well, large amounts of acetylcholine were then formed. We must therefore conclude that CrP in order to be effective as a phosphorylating agent in the synthesis of acetylcholine acts in combination with muscle adenylic acid, probably by the formation of ATP.

The reactivation of dialysed enzyme extracts by CrP with activator is shown in the following two experiments: in the one, the dialysed extract when incubated for 1 hr. with CrP alone synthesized 15 μg./g., but when incubated with activator as well it produced 360 μg./g./hr.; in the other, the corresponding rates were 30 and 360 μg./g./hr.

By comparison with the effect of activator, citrate combined with CrP and Mg ions had a relatively weak action and, as shown in Table 5, did not re-

TABLE 5. Formation of acetylcholine in *dialysed* saline extracts of acetone-dried brain. Effect of CrP with and without muscle and yeast adenylic acid or adenosine

	μg. acetylcholine formed in 1 hr./g. acetone powder		
Additions to samples	Exp. 1	Exp. 2	Exp. 3
Citrate	38	40	—
CrP, citrate	65	50	22
CrP, citrate, muscle adenylic acid	720	650	320
CrP, citrate, yeast adenylic acid	—	50	30
CrP, citrate, adenosine	—	50	25
CrP, citrate, activator	1200	—	—
CrP, citrate, activator muscle adenylic acid	1500	—	—

activate the dialysed extract to any extent. Thus only 22–65 μg./g./hr. were formed in samples incubated with CrP, citrate and Mg ions together. These values increased to between 320 and 850 when muscle adenylic acid was added as well.

The optimal effect of muscle adenylic acid is obtained with relatively low concentrations, since its action is again depressed with concentrations greater than 0·01 mg./c.c. adenylic acid-P. This result may be seen in Fig. 3, in which the different concentrations of muscle adenylic acid are plotted against the percentage increase of acetylcholine above that found in the absence of adenylic acid. Some of the points are the mean values of two or three observations.
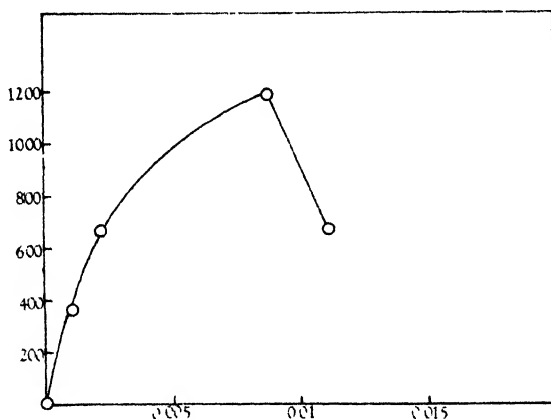
Fig. 3. Effect of varying concentrations of muscle adenylic acid on the formation of acetylcholine by *dialysed* saline extracts of acetone dried brain in the presence of CrP, citrate and Mg ions. Abscissae: concentration of muscle adenylic acid-P in mg./c.c. Ordinates: percentage increase of acetylcholine above level found in the absence of adenylic acid.

The effectiveness of muscle adenylic acid in increasing the synthesis of acetylcholine depends upon its action in combination with CrP. If muscle adenylic acid is given alone, or given with creatine plus inorganic phosphate, or given with ATP, it has no effect.

The effect of muscle adenylic acid is specific: it cannot be replaced by yeast adenylic acid or by adenosine (see Table 5).

DISCUSSION

Our experiments show that Mg ions have a great accelerating effect on the synthesis of acetylcholine, but only when the system includes citrate. In such a system, the presence of either Mg ions, or an ion which like Mn^{++} can replace Mg^{++}, is necessary to obtain the full effect which citrate can have. The effect of Mg ions has hitherto been overlooked; in earlier experiments it was examined on the synthesis of acetylcholine in the absence of citrate, and the effects of citrate were examined always in the presence of Mg^{++} only.

When synthesis of acetylcholine is studied in a system which includes the activator instead of citrate, the presence of Mg ions is of little importance. This suggests that the mechanisms of synthesis, the one involving activator, the other involving citrate are independent of one another. Any suspicion, therefore, that the activator can be identical with citrate is removed by these considerations, if the evidence given previously (Feldberg & Mann, 1946) were not already sufficient to show this.

Creatine phosphate (CrP) like ATP appears to be a normal constituent of nerves and brain (Gerard & Wallen, 1929; Holmes, 1933; Kerr, 1935). According to Kerr it is present in mammalian brain tissue in a concentration of between 0·08 and 0·14 mg./g. CrP-P. The optimal concentration for the synthesis of acetylcholine in our experiments was of the same order, i.e. 0·1 mg./c.c. CrP-P. Lohmann (1935) and Torres (1935) have demonstrated that the CrP metabolism in nervous tissue involves adenylic acid as a phosphate carrier, the ATP formed being the phosphate donator. It is probable that a similar interpretation can be given to our finding that, in dialysed saline extracts of acetone-dried brain tissue, CrP can act with citrate and Mg ions to replace ATP, only if muscle adenylic acid is added as well. It is also likely that our preparations of activator, which are obtained by boiling saline extracts of brain tissue, contain muscle adenylic acid since it is both heat-stable and dialysable, and that the formation of ATP when either activator or muscle adenylic acid as such is added, is the means by which CrP acts in the synthesis of acetylcholine.

SUMMARY

1. Mg ions accelerate the synthesis of acetylcholine by saline extracts of acetone-dried brain in the presence of citrate. In non-dialysed saline extracts Mg ions increase the synthesis 2-3 times, in dialysed extracts sometimes as much as 20 times. Without citrate, Mg ions have little or no effect. The strong accelerating action which citrate itself has on the synthesis of acetylcholine is, in fact, dependent on the presence of. Mg ions. Mg ions can be replaced by Mn, but not by Ca, Zn or Co ions.

2. Creatine phosphate (CrP) can replace ATP in the synthesis of acetylcholine by *non-dialysed* extracts prepared from acetone-dried brain.

3. CrP does not replace ATP in the synthesis of acetylcholine by *dialysed* extracts unless either activator or muscle adenylic acid is added as well. The effect of muscle adenylic acid is specific, and it cannot be replaced by yeast adenylic acid or adenosine. The theory is discussed that CrP, in order to act in the synthesis of acetylcholine, involves muscle adenylic acid as a phosphate carrier, according to the Lohmann reaction, the ATP formed being the phosphate donator.

Note added in proof. ATP sensitizes the frog rectus muscle to acetylcholine. This was observed by Torda & Wolff (1945) and later independently by Babsky & Minajew (1946). These

authors suggest that ATP may not stimulate the synthesis of acetylcholine but that the synthesis observed in brain extracts by Nachmansohn & Machado and by Feldberg & Mann may be nothing but this sensitizing effect of ATP. It is true that even the minute concentrations of ATP present in the diluted extracts used for the assay on the frog rectus muscle are sufficient to sensitize it to acetylcholine. When brain extracts which have been incubated with ATP are assayed against pure acetylcholine solutions the values obtained for acetylcholine are in fact somewhat too high (Feldberg & Mann, 1945). However, in the experiments of Feldberg & Mann as well as in our experiments, the brain extracts were assayed against acetylcholine solutions to which were added equivalent amounts of the same brain extract after it had been boiled for a moment in alkaline solution and then neutralized (see Feldberg & Mann, 1945). This treatment hydrolyses the acetylcholine but does not abolish the sensitizing action of ATP. We have found that known solutions of acetylcholine added to brain extract containing ATP can be determined quantitatively in this way. Therefore with the method of assay used by Feldberg & Mann and by ourselves the sensitizing effect of ATP is taken into account and does not interfere with the result. According to Torda & Wolff the sensitizing effect of CrP compared to that of ATP is only slight; it would therefore scarcely affect the result even if it were not taken into account in the assay of acetylcholine.

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THE EFFECT OF OESTRONE AND RELAXIN ON THE X-RAY APPEARANCE OF THE PELVIS OF THE MOUSE

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Separation of the symphysis pubis of the mouse can be demonstrated by X-ray photographs from the 13th day of pregnancy onwards, and it proceeds at a variable rate, averaging about 1 mm. a day until full term on the 19th or 20th day (Hall & Newton, 1946 a). The object of the following experiments was to obtain evidence as to whether a special substance, relaxin (Hisaw, 1926), was the responsible agent, or whether the separation could be accounted for by oestrogen (Gardner, 1936). Previous experiments (Newton & Lits, 1938) having suggested that the ovaries were indispensable for the normal process, oöphorectomized virgin mice were used as test animals for the effects of various combinations of oestrone, progesterone and pregnant rabbit serum (P.R.S.), stated to be rich in relaxin (Hisaw, 1926; Marder & Money, 1944). Extracts of P.R.S., prepared by the method of Abramowitz, Hisaw, Kleinholz, Money, Talmage & Zarrow (1942), are referred to for convenience as Relaxin I, II, III, etc. A preliminary statement of some of the results has been made (Hall & Newton, 1946 b).

In addition to these experiments on oöphorectomized mice, which confirmed the existence of relaxin, others were made on pregnant animals with the object of gaining insight into the physiological relationships of this substance. These also are recorded below. They confirmed the fact referred to above that normal pubic separation does not take place in the absence of the ovaries.

Some hundreds of X-ray measurements have been made and the results are presented as concisely as possible.

METHODS

Oestrone (B.D.H.) was weighed out, taken up in acetone, dissolved in groundnut oil (kept oxygen-free) and the acetone removed in vacuo. Dilutions of the stock solution were made with groundnut oil, so that the volume injected was 0·05 c.c.

Progesterone was used in the form of 'Proluton', 10 mg./c.c. of oil.

Pregnant rabbit serum (P.R.S.) was obtained by bleeding rabbits from the ear vein on or after the 27th day of pregnancy and allowing the blood to clot.

Extract of P.R.S. ('relaxin') was prepared according to the directions of Abramowitz *et al.* (1942). An equal volume of alcohol was added to P.R.S., the precipitate discarded, and the supernatant fluid treated with 9 vol. of acetone. The resulting precipitate contained the relaxin, and after centrifuging and discarding the supernatant fluid it was dissolved in a small quantity of water, sometimes with difficulty, yielding a brown turbid solution. The batches from Relaxin III onwards contained as nearly as possible 0·4 g. of the final precipitate (wet weight) per c.c. Relaxins I and II were made up by adding a volume of water one-tenth that of the original volume of serum, but the precipitates were not weighed. Assuming similar yields of precipitate, however, they were comparable in strength. The final volumes of all batches were between one-fifth and one-sixth of the original volume of serum.

X-ray examination of the pubis was made by the method previously described (Hall & Newton, 1946a). The use of a photographic enlarger enabled measurements to be made to 0·1 mm., but the quality of some of the photographs precludes our claiming this degree of accuracy throughout the series. All animals were examined immediately before the experiment to exclude abnormality of the symphysis.

Mated mice were examined every morning, and the midnight following the finding of a seminal plug was taken as the end of the first day of pregnancy.

RESULTS
Oöphorectomized mice

1. Three batches, each of six mice, received daily injections respectively of 0·025, 0·1 and 0·4 μg. of oestrone. There was no visible alteration in the symphysis pubis after eleven injections.

2A. Twelve mice were injected with 1·6 μg., six mice with 6·4 μg., and eleven mice with 25 μg. of oestrone, all daily. Six mice received daily 1·6 μg. oestrone + 0·5 c.c. non-pregnant rabbit serum (N.P.R.S.), and six mice daily 25 μg. oestrone + 0·5 c.c. N.P.R.S.

These mice were X-rayed at intervals, and there was no significant difference in the behaviour of the pubes as between the batches, although a slight opening was observed in all but a few of the animals. In all but two mice, measurements of the pubic gap were made after nine, ten or eleven injections. The mean increase in the gap for thirty-nine mice after this interval was 0·2 mm. (standard deviation ± 0·14 mm.; S.E. of mean ± 0·02).

2B. Nine mice received 1·6 μg. oestrone + 0·1 c.c. P.R.S., and eight mice received 25 μg. oestrone + 0·1 c.c. P.R.S., daily. The oestrone injections were started 2 days before those of P.R.S. After ten or eleven injections of oestrone (eight or nine of P.R.S.), the mean pubic gap in these seventeen mice was 0·93 mm. (standard deviation ± 0·52 mm.; S.E. of mean ± 0·13).

3A. In eleven of the mice (2A) receiving oestrone alone (six of the 6·4 μg. group and five of the 25 μg. group) the injections were continued and the pubic gap measured after sixteen injections. The mean separation was 0·36 mm. (standard deviation ± 0·2 mm.; S.E. of mean ± 0·06).

3B. In seven of the mice (2B) receiving 1·6 μg. oestrone + P.R.S., the injections were continued and the pubes examined after sixteen doses of oestrone

(fourteen of p.r.s.). The mean pubic gap was 1·4 mm. (standard deviation ± 0·6; s.e. of mean ± 0·23).

4. Preliminary experiments, made by W. H. N. some years ago, showed that a daily dose of 1 c.c. p.r.s. + 20 μg. oestrone produced in nine mice after 8 days an average pubic gap of 1·8 mm. This was measured directly in the killed animal. Oestrone injection was replaced by a 10–15 mg. implant of oestradiol in four mice, and the average gap after 8 days was 2·4 mm.

5A. The results obtained with concentrated relaxin are given in Table 1, and show respectively the effects of 0·1 and 0·2 c.c./day. They involve five batches, of which III, V, and VI are comparable in strength on the basis of the

TABLE 1. Increase in pubic gap in mm. in oophorectomized mice receiving daily 25 μg. oestrone + a dose of p.r.s extract (relaxin), indicated in column 1, on the 3rd and subsequent days of oestrone injection. The X-ray measurements were made on the day following the injection. The course of closure of the symphysis after the experiment is shown for five of the animals

Pubic gap (mm.)

Dose and batch of relaxin	Mouse	Days after start of relaxin injections (mm.)													Days after stoppage of injections		
		1	2	3	4	5	6	7	8	9	10	11	12	13	2	7	13
0 1 c.c. Relaxin I	E 122	1·6	—	2·1	—	2·3	—	..	2·6	—	2·8	.-	—	—	—	—	-
	E 123	0·7	—	0·8		1·4	—	..	1·6	—	2·8	--	—	—	..		...
	E 124	0·5	..	0·8	—	?	—	—	1·4	—	1·9	--	..	-	-	—	—
	E 125	0·4	—	1·6	-	1·7	—		2·1	—	2·2	--	—	—	--	...	-
0·1 c.c. Relaxin II	E 127	0·2	—	..	—	—	—	..	1·4	—	2·2	—	2·2	2·6		...	—
0·1 c.c. Relaxin III	E 129	0 1	-	—	—	0·9	—	1 5	—	2·2	—	2·6	3·0	·	—	...	..
0·2 c.c. Relaxin V	E 144	—	0 2	—	—	2·6	—	3·4	3·9	·	—	—	—	—		·	—
	E 145	—	0·2	—	—	2·3	—	3 4	4·0	—	·	—		·		—	—
	E 146	·	0·9	—	..	3·3	—	4·1	4·4	--	—	·		·		—	—
	E 147	—	0·8	-	—	2·9	·	3·8	4·6		...	—	—		—	--	..
	E 148	--	1·6	—	—	4·9	—	5 3	4·7	—	—	—	—		—	--	—
0·2 c.c. Relaxin VI	E 149	—	2·1	—	4·2	4·5	--	—	·	-	—	—	—	—	4·6	4·2	3·3
	E 150	·	0·2	—	1·7	3·2	—	..	4 2	--	—	—	—	·	2 2	1·2	0·9
	E 151	—	1·1	--	2·9	3 9	-	—	4·4	—	·	-	—	-	3 8	2 3	1·5
	E 152	·	0·7	—	1·7	3·1	—	—	2 9	--	·	...	—	—	1·7	1·3	0·7
	E 153	·	1·7	—	2·4	3·4	—	—	3·8	--	—	—	—	...	2·7	1·6	1·4
Average of 0 2 c.c. relaxin	—	—	0·9	.-	2·6	3·4	—	4·0	4·2	--	·	...	—	—		3·0	2·1	1·5

wet weight of the final precipitate. I and II are approximately comparable to the rest; more than one rabbit contributed to each batch. It is safe to conclude that, unlike oestrogen, relaxin exerts a greater effect with a greater dose.

5B. The pubes of three control animals, treated in the absence of oestrone with Relaxin VII (of the same strength as III, V and VI), separated respectively by only 0·1, 0·6 and 0·5 mm. after eight injections of 0·3 c.c. (1·5 times the dose given with oestrone). The vaginal smears of these mice were not cornified. The pubes of eleven mice receiving eight injections, and two mice receiving nine injections of 1 c.c. p.r.s. per day had separated by an average of less than 0·5 mm. in earlier experiments, including six in which the gap had been estimated by direct measurement.

6. The oestrone injections into the mice of Exp. 3 A were continued, and the five having the largest pubic gaps on the 22nd day of injection received in addition 1 mg. progesterone per day (in 0·1 c.c. oil) for 10 days. The average width of the gap in these mice on the 22nd day was 1·1 mm., and on the 31st day 0·9 mm.

The average gap of the whole group (3A) of eleven on the 22nd day of oestrone injection had risen since the 16th day from 0·36 to 0·72 mm. The standstill or regression of the pubic gaps of the five selected mice between the 22nd and 31st days therefore suggests, though it does not prove, that progesterone has an inhibitory effect on the separation.

7. Six mice received 1·6 μg. oestrone + 0·1 c.c. P.R.S. + 1 mg. progesterone daily. The oestrone injections were started 2 days before those of P.R.S. and progesterone. After thirteen injections of oestrone (eleven of P.R.S. and progesterone), the average pubic gap was 1·1 mm. Studied in relation to the results of 2 B and 3 B, this suggests that progesterone is without any marked influence.

Pregnant mice

8. It is worth recording, in support of the foregoing results, that, in some of the preliminary experiments made by W. H. N., pregnancy was terminated in sixteen mice by removal or dislodgement of the uterine contents on the 13th or 14th day, i.e. when the pubis was in its optimal reactive condition. The mice were killed and the pubic gaps estimated by direct measurement on the 20th or 21st day. These mice had all received a 10–15 mg. subcutaneous oestrone or oestradiol implant on the 12th day. Five received no other treatment, and the pubes were found movable with gaps of less than 0·5 mm. Six received in addition 1 c.c. N.P.R.S. daily and the gaps were 0·5 mm. or less. Five received 1 c.c. P.R.S. for at least 5 days, and the average gap was 2·3 mm.

9. Further experiments were made on pregnant mice to obtain some idea of the physiological relationships of relaxin.

(*a*) 1 mg. progesterone was injected daily into eight mice from the 14th day of pregnancy onwards in order to maintain pregnancy and their ovaries were removed on the 15th day. All carried foetuses until the 19th day or longer, and all showed diminution in size of the pubic gap beginning after oöphorectomy. The average gaps were: 15th day, 1·0, 17th day 0·55, 19th day 0·45, 20th day 0·32 mm.

(*b*) Seven mice, treated as in (*a*), also received oestrone daily from the 16th day—three had 1·6 μg. and four had 25 μg./day. The size of the gap remained practically constant after oöphorectomy in all the animals.

(*c*) Seven normal pregnant mice received 1·6 μg. oestrone daily from the 15th day and littered on the 19th or 20th day. The average gap at parturition (using, in each case, the greater of the 19th and 20th day X-ray measurements) was 4·0 mm., i.e. no greater than the normal average.

(d) Twenty normal pregnant mice received 0·5 or 1·0 mg. progesterone per day from the 15th day (in two the injection began on the 14th and in one on the 13th day). Parturition was delayed in many of these animals, and, in about half of them, the pubic gap continued to enlarge the whole time, but in others (Table 2) it had begun to close again before delivery. The average of the maximal gaps, irrespective of the day on which the maximum occurred, was 4·6 mm. This is again somewhat less than the normal average, and there was no significant difference between the responses of the subgroups.

TABLE 2. Partial closure of the symphysis pubis in certain mice in which parturition was delayed by 1 mg. daily of progesterone from the 15th day of pregnancy (in 450208 A the first injection was on the 13th and in 450214 on the 14th day). The measurements were made on the day stated

Mouse	Dose progesterone mg./day	Pubic gap (mm.) Day								Litter or post-mortem findings
		13	15	17	18	19	20	21	22	
450627 A	0·5	—	0·7	2·7	—	4·8	2·4	—	—	Litter found morning of day 21; 6 alive
450907	0 5	0·4	0·9	—	4·0	5·0	3·7	3 0	–	Littered during day 21. No further record
450908 C	0·5	0·4	2 2	4 4	—	6 0	4 8	—	—	Litter found morning of day 21; 10 dead
450908 I	0·5	0·3	0·3	1·1	—	2·7	2 4	—	—	Mouse killed day 21; 1 live foetus in utero + blood and detritus; 8 other placental sites
450908 J	0·5	0·2	0·7	3·2	—	5·3	3·6	—	.	Mouse killed day 21, 5 full-sized foetus in utero. Dead, but looked recently dead
450208 A	1·0	0·3	0·4	1·0	—	?	2·8	2·4	..	Litter found morning of day 22, 4 dead
450214	1·0	0 4	2·3	3·7	—	3·0	—	1·6	—	Mouse killed day 22; 3 macerated foetus in utero
450714 A	1·0	—	3·3	4·8	4·8	4·4	3·5	3 0	-	Littered during day 21 (after X-ray); 5 dead
450714 E	1·0	—	2·2	4·5	5·4	3·9	3·4	—	—	Litter found morning of day 21; 3 dead
450716	1·0	—	1·0	1·4	2·4	2 9	3·0	—	1·8	Litter found morning of day 22; 1 partly eaten

(e) The experiments of (d) were repeated in six mice in which the foetuses were destroyed on the 14th day of pregnancy, the placentae being left in situ. The average maximum pubic gap was 4·4 mm., and by the end of the experiment on the 22nd day, all the gaps had started to close. The delivery of the placentae was not observed.

The large number of experiments made under (d) and (e) were required because of two or three very large gaps (e.g. 6·5 mm.) which occurred in similar experiments in the preliminary series. We were unable to reproduce these results.

DISCUSSION

Twenty years ago, Hisaw (1926), using oestrous or oestrogen-primed spayed virgin guinea-pigs, showed that 8-24 hr. after an injection of pregnant rabbit or guinea-pig serum, the joints of the pelvis relaxed so that the two halves could be moved independently. To quote from a recent paper (Hisaw, Zarrow, Money, Talmage & Abramowitz, 1944) · 'The relaxative substance was found also in the blood of pregnant sows, dogs, cats and mares, in extracts of the placentae of rabbits and corpora lutea of the sow. Methods of extraction and concentration and the chemical properties were also studied...and the active agent was given the name relaxin.' The main findings were confirmed by various observers, but others found that an apparently similar effect could be obtained by the use of pure oestrogens, progesterone or combinations of these substances. In spite of claims by Hisaw and his colleagues that relaxin had no oestrogenic or progestational properties, some scepticism ensued as to the existence of this substance. The question was effectively settled by Abramowitz, Money, Zarrow, Talmage, Kleinholz & Hisaw (1944) who prepared relaxin in quantity from the corpora lutea of sows and showed that an amount 500 times greater than that required to produce a very definite positive response was without oestrogenic or progestational effect. Hisaw *et al.* (1944) further showed that the relaxation produced by progesterone was delayed, as compared with that produced by relaxin, and that it did not occur in hysterectomized guinea-pigs, whereas hysterectomy made no difference to the response to relaxin They therefore believe that progesterone causes the production of relaxin through the medium of the reproductive tract, and they adduce the additional evidence that the blood serum of non-pregnant rabbits can be rendered active by injections of oestrogen and progesterone or oestrogen alone provided the uterus is present, but not otherwise. References to the fairly extensive literature bearing on the action of oestrogens, progesterone and relaxin on the guinea-pig are given in the two papers cited

With regard to the action of oestrogen, Hisaw and his colleagues recognize that large doses given for a long period will produce bony resorption and ligament formation at the guinea-pig symphysis, but the brief priming with oestrogen which is necessary for the action of relaxin has no such effect. They do not rationalize the three apparent effects of oestrogen, the priming effect, the separate action on the symphysis and the apparent ability to induce the secretion of relaxin in the presence of the reproductive tract. With regard to relaxin, its claim to individuality rests chiefly on the speed of its action, its chemical difference from the other two hormones, and its action in the hysterectomized animal.

The conclusions to be drawn from our experiments on the mouse are as follows: Groups 1, 2A and 3A show that oestrone, above a certain dosage, will effect a separation of the symphysis pubis and that the longer it is administered the greater is the separation. There was statistically no significant difference between the responses of the subgroups of 2A, while group 9 (c) shows that an otherwise effective dose of oestrone does not potentiate the process occurring in normal pregnancy. Although oestrone is rapidly absorbed and excreted (Parkes, 1937), the effect of single doses of 2 μg. persists for 4 days in mice (Emmens, 1939). Our daily injections of 1·6 μg. and over would therefore maintain the blood concentration. but it is hard to say whether this increased with increasing dosage, or was maintained saturated. Emmens's further observation that a dose of 200 μg. was effective for only 7 8 days argues considerable powers of absorption. Whatever the explanation. there was no increased separation of the symphysis on increasing the effective dose, and while the observations of Gardner (1936) on the action of oestrogen are confirmed, the

rate of separation is negligible compared with the 1 mm./day which is achieved during pregnancy (Hall & Newton, 1946a).

The experiments of group 4 show that the administration of 1 c.c. P.R.S./day in addition to oestrone greatly enhances the rate of separation, and those of group 5 (Table 1) that by increasing the dosage of the active material contained in P.R.S. a rate of response not far short of that occurring in pregnancy can be obtained. Within the range of dosage employed, the effect increases with the amount of P.R.S. or its extract given, and the method of extraction is that prescribed by Abramowitz et al. (1942) for the concentration of relaxin.

The responses of the mice in groups 2 B and 3 B were significantly greater than those of their controls in groups 2 A and 3 A. The value of 't' for the difference between the means of groups 2 A and 2 B is 8·12 and for that between the means of groups 3 A and 3 B, 5·54, i.e. the chance that the difference is not significant is negligible. The amount of active material in 0·1 c.c. P.R.S. can therefore be easily detected; smaller quantities were not tested. This is the dose which gives a positive response in about 66% of guinea-pigs (Marder & Money, 1944), so that as a method of testing for or assaying relaxin, our procedure would probably offer little advantage except that of providing an objective record.

The experiments of groups 6 and 7 show that progesterone has no potentiating action on the response, and that it might be inhibitory to the oestrone-alone reaction. Groups 9 (d) and 9 (e) are confirmatory, progesterone having no detectable influence on the normal process as it occurs during pregnancy. We cannot, however, claim finally to have excluded a secondary action, such as that suggested by Hisaw et al. (1944), until we have given progesterone earlier in pregnancy, because it takes 3 days to act indirectly in the guinea-pig.

Groups 9 (a) and 9 (b) confirm the earlier conclusion (Newton & Lits, 1938) that the ovaries are necessary for the separation as it occurs in pregnancy. Group 9 (b) offers no suggestion that the deficiency could be repaired by oestrone and progesterone. Hisaw (1929), however, states that relaxation progresses after removal of the ovaries in the pregnant guinea-pig and, that P.R.S. retains its properties after the rabbit has been oöphorectomized, provided in each case the animals do not abort.

Group 9 (e) confirms that so far as the products of conception are concerned the placenta is the essential component and that it acts in the presence of added progesterone. Table 2 shows that when delivery is delayed by progesterone the pubic gap may begin to close while the uterus still retains its contents. These two observations mean that the mere presence of material in the uterus is an unimportant factor, a point hitherto difficult to prove. All the mice of group 9 (d), whose pubic gaps continued to enlarge until parturition, had live litters, and most of those whose gaps began to close had dead litters, either

delivered or found at autopsy. But 450627 A had a live litter, 450908 I had one live foetus (though the closure of the gap was slight), and the foetus of 450908 J looked as though they had died very shortly before autopsy on the 20th day. The state of the litter 450907 was not recorded. (The experiments were aimed at establishing the maximum gap, and the importance of the secondary data was only appreciated in retrospect.) Therefore, although death or separation of the placenta is the most obvious cause of the gap beginning to close in these circumstances, we cannot exclude some other factor.

GENERAL CONCLUSIONS

Our results clearly confirm the existence of relaxin. Although we have not thoroughly tested P.R.S. or its extract for oestrogenic or progestational activity, we have shown that doses of oestrone and progesterone in excess of what it could possibly have contained are without significant effect.

With regard to its mode of action, we have as yet no data to prove that relaxin is not essentially a potentiator of the action of oestrogen on the symphysis. The potentiation is considerable, and the slight effect of P.R.S. alone in oöphorectomized animals could be due to traces of oestrogen from non-ovarian sources. Histological comparison of the interpubic ligaments produced by oestrone alone and in combination with relaxin has not yet been made, but at present it seems just as likely that relaxin potentiates the action of oestrogen as that its own action on the symphysis is the fundamental one, though it requires the presence of oestrogen.

With regard to the source of relaxin in the pregnant mouse, this and other investigations have shown that the placenta is undoubtedly concerned. Since only two substances are required for the reaction, and since oestrogen could be contributed by the ovary, the question reduces to whether the placenta contributes relaxin itself or causes the ovary to do so. Our experiments point to the ovary as the source, and this would be in accordance with the occurrence of relaxin in the corpus luteum of the sow and with the known luteotrophic action of the mouse placenta. Both the luteotrophic action of the placenta and the separation of the pubes are independent of the presence of the pituitary (Deanesly & Newton, 1940; Newton & Beck, 1939; Gardner & Allen, 1942). Relaxin has, however, also been found in the rabbit placenta (Hisaw, 1927), and Hisaw *et al.* (1944) suspect that it can be produced by the action of progesterone on the uterus. The importance of the uterus and its contents in the pregnant mouse is clear, but our experiments with progesterone have been negative. In the experiments of group 9 (*b*) enough progesterone was present to maintain pregnancy in the absence of the ovaries, and the dosage of oestrone had been shown to be effective in conjunction with rabbit relaxin. If the placenta had been secreting relaxin, its presence should have been manifest.

If, as Hisaw (1929) states, the ovaries of pregnant rabbits and guinea-pigs are not essential for its production, a species difference may be involved.

Whatever the source of relaxin in the mouse, the abrupt onset of its action on the 14th day of pregnancy remains to be explained.

SUMMARY

1. Daily injections of oestrone cause a slow opening of the symphysis pubis of the spayed mouse which increases with the duration of treatment, but which is independent of dosage above 1·6 μg./day.

2. This oestrone-alone effect is increased twentyfold by the daily injection of 0·2 c.c. of an extract of pregnant rabbit serum stated (Abramowitz *et al.* 1942) to contain relaxin. The effect of 0·1 c.c. daily of unconcentrated pregnant rabbit serum can be readily detected, and is assumed to be due to relaxin. The effect of the latter in the presence of oestrogen appears to increase both with the daily dosage and with the duration of treatment.

3. Relaxin has only a slight effect in the absence of administered oestrogen.

4. Increasing the dosage of oestrone beyond 1·6 μg./day does not enhance the oestrone-relaxin effect, and injected oestrone does not accelerate the normal process in pregnancy. It is doubtful which substance exerts the fundamental action on the symphysis and which is the potentiator.

5. Progesterone up to 1 mg./day (for the durations of treatment employed) does not potentiate the oestrone-alone effect, the oestrone-relaxin effect, or the naturally occurring process in pregnancy.

6. Oöphorectomy during pregnancy arrests the separation of the pubes even when 1 mg. progesterone + 25 μg. oestrone is administered daily.

7. The role of the products of conception in this process can be played by the placenta alone, but not by the mere presence of material in the uterus. An internal secretion of the placenta is therefore probably concerned.

8. The placenta may itself contribute relaxin, or evoke its formation by the ovary. The present experiments suggest that the latter occurs in the mouse, and the point is discussed.

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ANTIDIURETIC HORMONE IN PITUITARY GLANDS OF NEW-BORN RATS

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The suggestion has been made in a previous paper (Heller, 1944) that the apparent inability of new-born infants to concentrate the urine to the same degree as adults (McCance & Young, 1941) may be due, or partly due, to an inadequacy of the posterior pituitary control. Such an inadequacy may be caused by either a lack of response of the infantile kidney cells to the circulating antidiuretic hormone, or insufficient production of the antidiuretic principle by the posterior pituitary lobe, or both. An investigation of the first of these possibilities (Heller, 1944) showed that doses of posterior pituitary extract which produced a pronounced inhibition of water diuresis in adults had only a slight and fleeting effect on the kidney of new-born infants. The present series of experiments was planned to investigate the secretion of antidiuretic hormone immediately after birth.

Estimations of the posterior pituitary hormone concentration in the blood of new-born infants or animals would be the procedure of choice but the amount of analytical material obtainable would be too small to permit reliable estimations of the hormone(s). As a first approach, it was therefore decided to determine the antidiuretic hormone content of the pituitary gland of new-born rats.

METHODS

The pituitary glands of seventy-seven male albino rats, 18–42 hr. old, were used for the preparation of extracts. The animals were killed by decapitation within 2 hr. of separation from the mothers and the pituitary glands were removed with the aid of a binocular dissecting microscope. The whole pituitary gland was removed with a pair of fine forceps. The same forceps was used to clean the pituitary cavity with a small swab of cotton wool. The swab, and a thin slice of the brain tissue adjacent to the pituitary gland (containing the infundibular portion of the pars nervosa) were added to the material for extraction.

Weight of the whole pituitary gland of new-born rats. With the help of the dissecting microscope the gland was transferred to a weighed coverslip; the coverslip was placed in a tared weighing bottle and the weighing bottle weighed on a Sartorius micro-balance. The following typical weights were obtained for new-born rat pituitary glands (body weight of animal in brackets): 0·64 mg. (5·89 g.), 0·56 mg. (5·26 g.), 0·61 mg. (6·01 g.), 0·57 mg. (5·35 g.) and 0·63 mg. (6·02 g.).

Weight of pars nervosa of adult rats. With the help of low magnification the neurohypophysial tissue caudal to the pituitary stalk was separated from the pars glandularis and weighed in the same manner. Attempts to separate the pars nervosa of new-born rats were unsuccessful. The following weights were obtained for the pars nervosa of male adult rats (body weight of animals in brackets): 1·04 mg. (227 g.), 1·33 mg. (242 g.), 1·31 mg. (205 g.), 1·21 mg. (205 g.), 1·52 mg. (285 g.) 1·42 mg. (264 g.) and 1·26 mg. (225 g.). These figures agree with those of Degener (1922) but are somewhat higher than those of Kuschinsky & Liebert (1939).

Preparation of new-born rat pituitary extracts. Several glands were pooled for extraction. Extracts were made by macerating the glands in a solution containing 0·9% NaCl and 0·25% acetic acid. For each gland 0·3 c.c. of solution was allowed. The extracts were boiled for 3 min., were then filtered and the residue washed twice with 0·5 c.c. of 0·9% NaCl solution. Such extracts, termed 'saline extracts', were assayed on the day of preparation. It was found that pituitary extracts prepared in this manner frequently contained considerable amounts of histamine-like impurities. It was therefore decided to treat the material with acetone and to dry it according to the recommendations of the *British Pharmacopoeia* (1932a) before extracting with acetic acid Extracts prepared in this manner, termed 'acetone extracts', contained little or no trace of the histamine-like substances. The same procedure was used for the preparation of extracts of adult pars nervosa tissue. Extracts of brain cortex of new-born rats were used for control observations.

Assay of antidiuretic activity. Intravenous injections into unanaesthetized rabbits were employed (Heller, 1942).

Assay of oxytocic activity Virgin guinea-pig uteri were used.

The alkali-resistance of posterior pituitary-like principles in extracts from the new-born rat pituitary. The pharmacopoeial test (*British Pharmacopoeia*, 1932b) was employed.

Posterior pituitary extract (British Drug Houses) was used as the standard preparation.

RESULTS

Extracts from pituitary glands of new-born rats

The antidiuretic activity. Fig. 1 (A, B) shows how the antidiuretic hormone content per gland was estimated. The figures in Table 1 represent the antidiuretic activity per gland as estimated by assay of the extract from a number of pooled new-born rat pituitaries. There seemed to be no difference between the antidiuretic activity of 'saline' and of 'acetone' extracts suggesting that the presence of histamine-like impurities in the former did not interfere with the antidiuretic assay. Control experiments showed that histamine acid phosphate given intravenously in doses up to $15\mu g./kg.$ did not influence the water diuresis of rabbits. It will be seen from Table 1 that the antidiuretic activity of a single new-born rat pituitary gland equalled approximately 2 mu. (mu. = milliunit) of the standard posterior pituitary extract and Fig. 1 (C) demonstrates that this activity disappeared after treatment of the extract with cold alkali.

The oxytocic activity. Acetone extracts only were used for oxytocic assay. Five experiments, with extracts from twenty-six glands, gave uniform results. The oxytocic activity per gland was found to be equivalent to more than 2 and less than 5 mu. of the standard posterior pituitary extract. All, or almost all, of this oxytocic activity was destroyed by cold alkali.
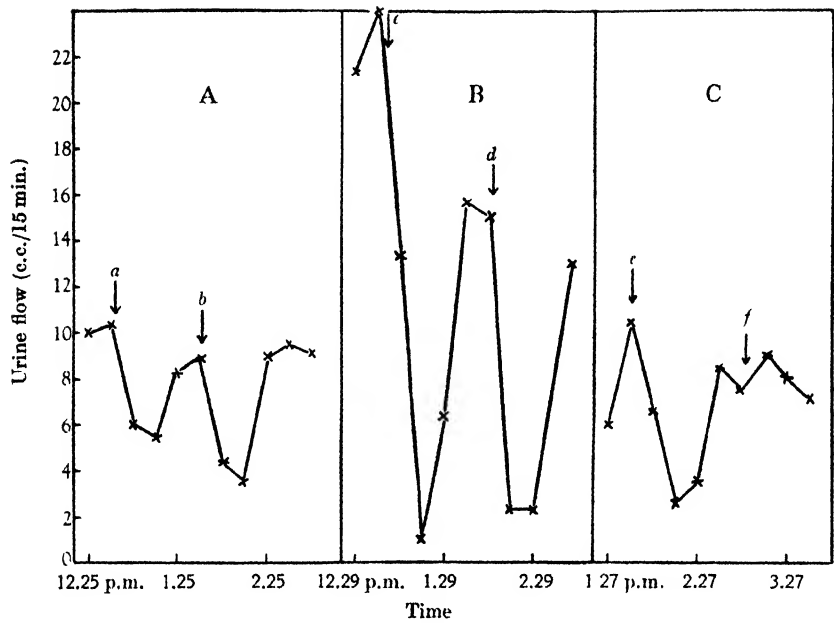
Fig. 1. Estimation of antidiuretic activity of new-born rat pituitary gland (A and B) and loss of antidiuretic activity of new-born rat pituitary extract after treatment with cold alkali (C). A, rabbit No. 26. At 10.05 and 11.45 a.m. 4 % of body weight of water given by stomach tube. *a* = intravenous injection of a dose equivalent to the extract of one-third of a new-born rat pituitary gland. *b* = 1·0 mu. of standard posterior pituitary extract intravenously. B, rabbit No. 27. At 10.09 and 11.49 a.m. 4 % of body weight of water given by stomach tube. *c* = intravenous injection of a dose equivalent to the extract of two-thirds of a new-born rat pituitary gland. *d* = 1·0 mu. of standard posterior pituitary extract intravenously. The antidiuretic activity of a single new-born rat pituitary gland was, therefore, equivalent to less than 3·0 and more than 1·5 mu. of the standard posterior pituitary preparation. C, rabbit No. 33. At 10.12 a.m. and 12.52 p.m. 4 % of body weight of water given by stomach tube. *e* = intravenous injection of a dose equivalent to the extract of one new-born rat pituitary gland. *f* = intravenous injection of extract of one new-born rat pituitary gland after treatment with cold sodium hydroxide.

TABLE 1. The antidiuretic hormone content of extracts from pituitary glands of new-born rats

No. of glands used for preparation of extract	Type of extract ('saline' = S, 'acetone' = A)	Antidiuretic activity per gland in terms of milli-units of standard posterior pituitary extract	
6	S	<5·0	~2·5
5	S	<2·0	<2·0
5	S	<2·0	
8	S	<2·0	>1·0
2	A	≪6·0	
2	A	≪5·0	
2	A	≪5·0	
4	A	>2·0	
9	A	<2·5	
2	A	~2·5	
2	A	<2·5	<2·5
4	A	<3·5	>1·5

The results of the oxytocic assay agree well with the estimations of antidiuretic activity. Considering also the inactivation by alkali, the antidiuretic substance extracted from the glands of new-born rats may be identified with the posterior pituitary antidiuretic hormone.

Extracts from the pituitary glands of adult rats

Four experiments with acetone extracts of the pars nervosa of adult rats showed an antidiuretic hormone content of more than 600 and less than 900 mu./mg. of pars nervosa tissue. The hormone content per gland was therefore more than 750 and less than 1100 mu. The results agree with earlier estimations (Heller, 1941) and suggest that the presence of pars glandularis tissue (the whole pituitary gland was extracted in those experiments) did not interfere with the antidiuretic assay.

Comparison of the antidiuretic hormone content of the pituitary gland of new-born rats with that of the glands of adult animals

The pars nervosa forms approximately 11 % (by weight) of the pituitary gland of the male albino rat (Stein, 1933a) and further data of this author (Stein, 1933b, 1934) indicate that the ratio pars nervosa/pars glandularis remains independent of the age of the animal. The weight of the whole pituitary gland of new-born rats, as found in the present series, was 0.60 ± 0.04 mg. New-born rat pituitary extracts contained less than 2.5 mu. of the antidiuretic hormone per gland (Table 1) and therefore, if Stein's data are used, less than 26 mu./mg. pars nervosa tissue. The pars nervosa of male adult rats of the same strain was found to contain an average of 750 mu. mg. Thus the pars nervosa of the new-born rat contains much less antidiuretic principle than that of the adult.

The mean body weight of the new-born rats used in the present series was 5.12 ± 0.73 g.; the mean body weight of adult rats, whose pituitary glands had been used for antidiuretic hormone estimations in the present and in an earlier (Heller, 1941) investigation, was 263 ± 54 g. It follows that the antidiuretic hormone content was approximately 38 mu./100 g. rat in new-born rats and 350 mu./100 g. rat in adult rats. When referred to body weight, the gland of new-born rats contained therefore about one-tenth of the antidiuretic principle contained in the adult gland.

Shanklin's histological studies have shown that the pars nervosa of new-born infants (Shanklin, 1940) and that of foetal pigs just before birth (Shanklin, 1944) are still in a primitive state of development. Rats are less mature at birth than the new-born of these two species. It seems justifiable, therefore, to attribute the low hormone content to a hypofunction of the posterior pituitary gland.

SUMMARY

1. Pituitary glands of new-born rats were found to contain the following amounts of active principles per gland: $>1<3$ mu. in terms of antidiuretic activity and $>2<5$ mu. in terms of oxytocic activity. Both activities disappeared after treatment of the extracts with cold alkali.

2. Calculated per 100 g. body weight, pituitary glands of new-born rats contained approximately one-tenth of the antidiuretic principle found in the glands of adult rats of the same strain.

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THE EFFECT OF SEVERE BURNS AND SOME PROTEIN-PRECIPITANTS ON SKIN-HISTAMINE IN CATS

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In previous experiments (Dekanski, 1945) it was found that moderate cutaneous burns in mice caused the formation of histamine, mainly in the skin. The total amount of histamine in the mouse was almost doubled in 10 min. and reached its maximum concentration within 1 hr. or less. If the mouse survived, the excess histamine was excreted in the urine during the next 48 hr.

These results confirm those of Kisima (1938) and Lambert & Rosenthal (1943), but not those of Harris (1927). Harris found that when the skin of an anaesthetized cat was burned with a hot flat-iron there was oedema, but no change in the histamine concentration in the burnt area of skin, during the first hour. After this time, he found gradual absorption of the histamine originally present, along with the oedema fluid. It was decided to investigate the discrepancy between the results obtained in this laboratory and those of Harris by performing experiments in which the histamine content of skin in cats was followed after moderate and severe burns.

Experiments were also carried out to examine the effect of various substances, which have been used in the treatment of burns, on the histamine content of normal and moderately burnt skin.

METHODS

The cats used were prepared on the previous day by shaving each flank. They were anaesthetized with sodium pentobarbitone ('Veterinary Nembutal', Abbott Laboratories Ltd.), given intravenously after induction with ether. Three or four areas were then outlined on each flank by means of a rubber stamp, so as to obtain six or eight patches of skin of equal surface area. Each of the outlined areas, except one or two controls in each experiment, was then burned at 60° C. or 80° C. for 60 sec. by means of the apparatus described by Leach, Peters & Rossiter (1943), or by means of a hot metal rod at 140° C. for 15 sec.

In the second series of experiments, burning was done at 60° C. for 60 sec. only. and most of the burnt areas, in addition to some unburnt areas, were treated with chemical solutions. The substances examined were 20% tannic acid, 10% silver nitrate, 2% gentian violet, 1% formalin (i.e. 1% solution of 40% formaldehyde) and 2% 'Mercurochrome', and were applied by a single, unrepeated painting, 10 min. after burning. One or two 'burnt controls' and 'unburnt controls' were left untreated.

In all these experiments after different periods, the patches of skin were removed, weighed, extracted with trichloroacetic acid (Barsoum & Gaddum, 1935), and boiled for 90 min. in conc. HCl (Code, 1937). The rest of the procedure was as originally described.

The effect in vitro of the substances on histamine itself and on the biological assay was tested by adding 150 μg. histamine phosphate to 1 c.c. of each solution and then putting the solution through the treatment with trichloroacetic acid, etc.

Estimation and identification of histamine was carried out by testing the extracts on a strip of guinea-pig's ileum suspended in 2 c.c. of Tyrode's solution containing atropine (0·1 μg./c.c.), and by intravenous injection into atropinized cats, anaesthetized as described above. The effects in all cases were compared with those obtained with standard solutions of histamine phosphate and the concentrations of histamine present were calculated in terms of histamine base. Blood-pressure readings often gave lower estimates of the histamine content than those obtained with the guinea-pig's gut. The discrepancy was never more than about 50% and usually much less and may have been due to experimental error. The results of all tests were in general agreement, and there can be little doubt that histamine was the main active principle.

Since burning alters the size and weight of the skin, results are calculated on the basis of the areas originally mapped out for treatment.

RESULTS

Comparison of moderate and severe burns. The individual results of these experiments are given in Table 1, from the averaged results of which Fig. 1 has been constructed. The average of the weight and diameter of the outlined areas are shown graphically in Fig. 2 in which each point, except for 140° C., represents the mean of five observations.

Burning at 60° C. was found to cause an intense congestion of the skin, and very intense oedema of the subcutaneous tissues within 1 hr. The extractable histamine equivalent in the skin rose to 22·6 from the average control amount of about 10·5 μg. per outlined area. A marked increase in histamine content was observed after 10 min., the maximum value being reached within 3 hr. The histamine equivalent was still high after 5 hr.

Burning at 80° C. was found to cause a contraction and thickening of the skin and moderate congestion and oedema. The extractable skin histamine dropped from about 10·5 to 9 μg. per burned area within 1 hr., and to 4·3 μg. after 5 hr.

Burning at 140° C. for 15 sec. caused the skin to become more thickened and contracted and to appear coagulated. The subcutaneous tissues initially showed little oedema, but this became evident in the surrounding area after an interval of 30 min. or more. The histamine equivalent dropped to 9 μg. per burned area within 1 hr., and to 2·6 μg. after 5 hr.

Effect of chemicals on normal and burnt skin. The individual results of these experiments are given in Table 2, from the averaged results of which Fig. 3 has been constructed. The average of the weights is shown graphically in Fig. 2 in which each point, except for 'Mercurochrome', represents the mean of five observations. The surface area in all cases was the same as in untreated burns caused by 60° C. for 60 sec.
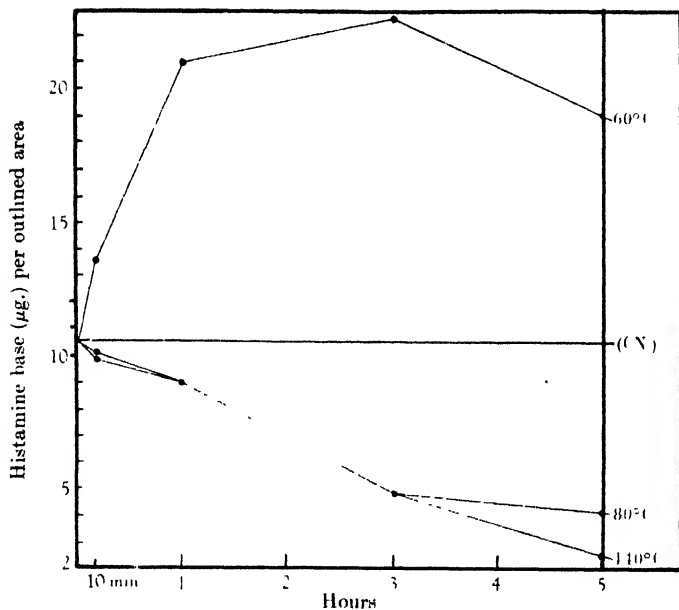
Fig 1. Histamine equivalent at different times after burning. CN value for normal skin.
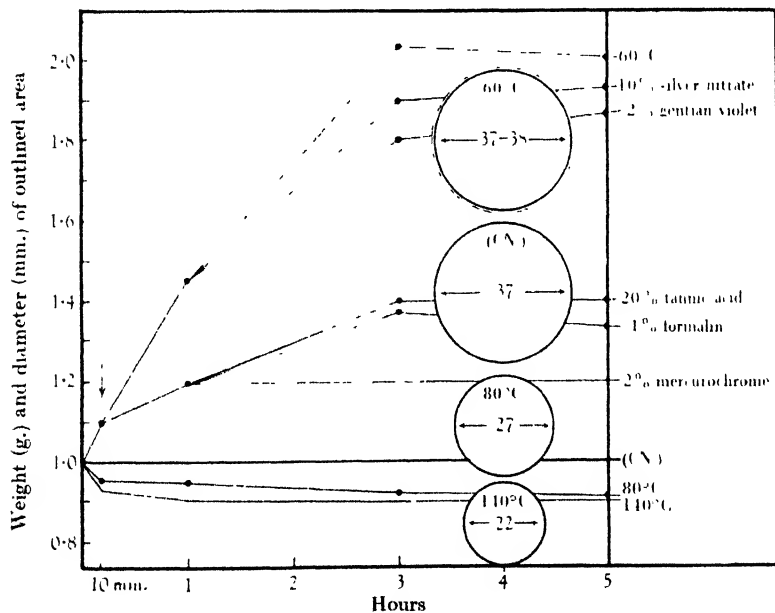
Fig. 2. Effect of burning and treatment on weight (graph) and area (circles) of cats' skin. Burns at zero time; treatment at 10 min. Burning at 60° C. increases the weight but not the area compared with unburnt controls (CN). The surface area in all cases treated was the same as in untreated burns caused by 60° C. Treatment with tannic acid, formalin and 'Mercurochrome' inhibits increase of weight. Silver nitrate and gentian violet were comparatively ineffective. Burning at 80° or 140° C. decreases the area definitely and the weight slightly.

3—2

TABLE 1. Individual results. Histamine equivalents (μg.) per area of control skin (CN) and burnt skin at 60° C. and 80° C. for 60 sec. and 140° C. for 15 sec. Extracts prepared chemically

Time after burning	60° C.		80° C.		140° C.	Mean (CN)
	I	II	I	II	II	
1 min.	10·0	10·6	10·6	11·0		
	9·6	10·5	10·0	11·3		
	9·0	10·6	8·3	10·6		
	9·3	11·6	8·7	11·3	11·8	
Mean (CN)	10·1		10·2			10·5
	10·0	*10·6*	*10·6*	*10·9*	—	
10 min.	11·5	13·7	10·0	11·0		
	11·5	17·0	10·7	11·3		
	11·6	14·8	8·0	10·6		
	10·8	16·1	—	8·0	10 2	
Mean (CN)	13·4		9·9			10·2
	9·0	*10·6*	*10·6*	*10·6*		
1 hr.	20·0	20·0	—	10·5		
	17·0	23·4	10·6	10·7		
	15·6	25·3	—	8 0		
	18·0	27·9	7·0	7·9	9·0	
Mean (CN)	20·9		9·0			10·4
	9·4	*10·6*	*10·7*	*10·9*	—	
3 hr.	21·5	28·4	—	5·3		
	—	21·7	—	4·5		
	17·3	25·9	5·6	3·8		
	17·7	25·9	—	5·2	4·9	
Mean (CN)	22·6		4·9			*11·2*
	—	*11·2*	—	*11·2*	—	
5 hr.	17·2	17·2	—	3 2		
	14·5	20 2	—	3·4		
	17·4	28·7	5·5	4·5		
	15·7	20·4	—	4 8	2·6	
Mean (CN)	18·9		4·3			*11·2*
	—	*11·4*	—	*11 0*	—	

I = Cat's blood-pressure test.
II = Guinea-pig's ileum test.
(CN) = Control unburnt.

The 20 % solution of tannic acid caused diminution of congestion and oedema in the burnt skin and a significant fall in the extractable histamine equivalent to 5·8 μg. per outlined area within 3 hr., as compared with 22 μg. in burnt controls at this time and with about 10·5 μg. in normal skin. The 10 % silver nitrate did not stop the development of oedema in burnt skin. An increase in histamine was observed after 1 hr., reaching a maximum value of 24·8 μg. per outlined area within 5 hr., an even higher value than in untreated burns. After treatment with 2 % gentian violet, 1 % formalin and 2 % 'Mercurochrome' the extractable skin histamine dropped to 11·1, 9·2 and 11·7 μg. per outlined area, approximately the figures for normal skin. After treatment with gentian violet, however, there was still intense oedema. Formalin appeared to cause pain

when applied to burnt skin. The results with 'Mercurochrome' are uncertain and incomplete, as it interfered with the biological test used in the experiments.

Similar results were obtained for the histamine equivalent of control unburnt skin similarly treated with each substance. Tannic acid decreased the histamine value of normal skin, while silver nitrate increased it to about the same extent as in burnt skin, as can be seen from Fig. 3. The other substances caused no distinct change.

TABLE 2. Individual results. Histamine equivalents (μg.) per area of control skin (CB). (CT), (CN), and burnt skin at 60° C. for 60 sec. treated with the astringent solutions and 'Mercurochrome'. Extracts prepared chemically

Time after burning or treatment	20% tannic acid		10% silver nitrate		2% gentian violet		1% formalin		2% mercurochrome		Mean control
	I	II	I	II	I	II	I	II	I	II	
1 hr.	4 1	6 6	14·1	23 2	8·3	11·2	6·0	11·4			
	5·7	4 5	20 7	33·1	--	9·7	7·1	9·0			
	---	7·7	--	24 8	10 3	11·7	10·1	—			
	4·0	7 2	20·7	23 1	10·3	10 3	8 2	9·6			
	3 8	6 3	20 4	27·1	10·2	12·5	9·0	10·7	12 1	11 4	
Mean	5·5		23·0		10·5		9·0		11 7		
3 hr.	4·6	7·3	14·8	28 7	10 8	10·5	6 0	11·7			
	5 7	5·0	20·7	34·4	11·8	11·9	7·2	9·1			
	—	7 8	--	25 1	10·3	10 3	10 2	—			
	4·1	7·4	21 1	23·8	10 2	12·4	8 2	9·6			
	3·9	6·5	20·7	28 5	—	--	9·0	10·0	12·0	11 4	
Mean	5·8		24 2		11 0		9·0		11·7		
(CB)	*20 0*	*20·0*	*21·5*	*28·4*	*19·6*	*26 0*	*22·3*	*18 2*	—	—	*22 0*
5 hr.	4·6	7·4	17·3	28 5	10 8	10 2	6·8	12·0			
	5·8	5·4	21 4	34·2	12 0	12·0	7·4	9 1			
	--	7·7	—	25·6	10 4	10·4	10·1	—			
	4·1	6·5	17·0	23·4	10 4	12 7	8·0	9 5			
	3 9	5 5	22 9	33 0	---	—	9 0	10·9	12·1	11·4	
Mean	5·6		24·8		11·1		9 2		11·7		
(CB)	*17·2*	*17·2*	*17·4*	*28·7*	*24 8*	*26 4*	*14·6*	*16·0*	—	—	*20·0*
(CT)	*3·4*	*3·2*	*20·1*	*20·1*	*10 0*	*10 0*	*10·2*	*10·7*	*10 0*	*9·5*	
(CN)	*10·0*	*10·8*	*10 0*	*10·9*	—	*10·0*	*10·0*	*10·0*	--	*10·0*	*10 2*

I = Cat's blood-pressure test. (CT) = Control unburnt, but treated.
II = Guinea-pig's ileum test. (CN) = Control neither burned, nor treated.
(CB) = Control burnt only.

Experiments in vitro. The results in vitro (six results in each case) showed 100% recovery of histamine added to tannic acid and to gentian violet, and 98% of the histamine added to formalin. Kendall (1927) discovered that histamine is inactivated by formalin in vitro with the formation of a condensation product of histamine and formaldehyde. Best & McHenry (1931) found that formaldehyde produces an immediate effect on a solution of histamine, causing a loss of approximately 50% of its pharmacological acitivity, followed

by a slow secondary reaction in which the balance of the activity is lost if the histamine-formaldehyde solution is kept at room temperature aseptically for 3 weeks. The histamine activity lost through the formation of a condensation product is, however, almost completely restored by boiling the mixture with hydrochloric acid. Hence formalin does not actually destroy the histamine, except after a long time, and this probably accounts for the almost quantitative recovery obtained by the present procedure.

Histamine added to silver nitrate solution could not be recovered by the extraction process.
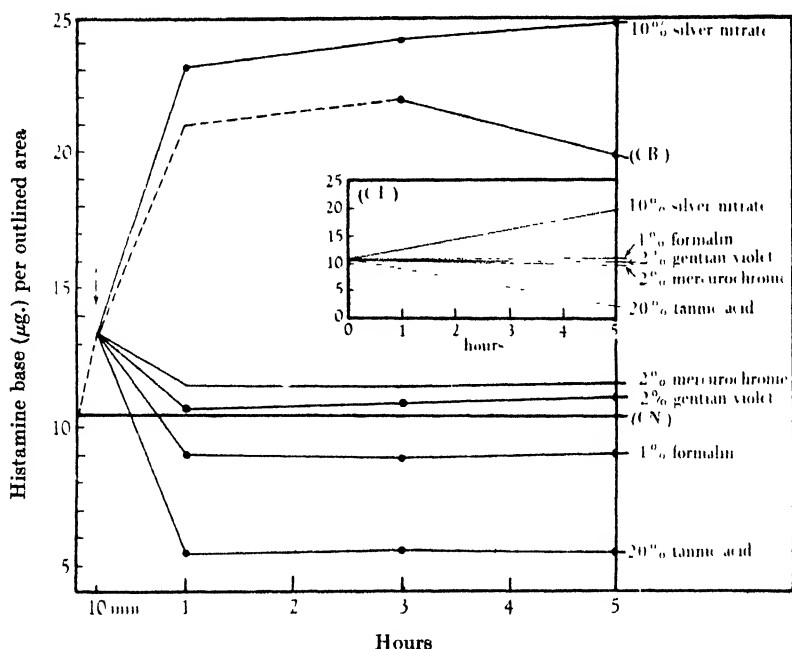
Fig. 3. Histamine equivalent at different times after treatment. Burns at zero time; treatment at 10 min. Tannic acid decreases extractable histamine equivalent in burnt skin, while silver nitrate increases it compared with values of normal skin (CN) and untreated burnt controls (CB). With other substances the values were approximately the same as in normal skin. The small inserted figure (CT) shows similar results with unburnt skin treated with each substance.

These controls suggest that the results with tannic acid, gentian violet and formalin were reliable. The results with silver nitrate render the in vivo experiments difficult to interpret. It seems that the figures obtained should be regarded only as minimal. However, the presence of protein in the animal experiments probably greatly retarded destruction of histamine by silver nitrate.

With a solution of 'Mercurochrome', although all added histamine appeared to be recovered, the compound affected both the guinea-pig's ileum and the

anaesthetized cat's blood-pressure, so that a few minutes after the first test, the preparation became abnormal in response to histamine. Thus proper assays were impossible.

<center>DISCUSSION</center>

The application of heat and of various chemicals has marked effects on the amount of histamine which can be extracted from skin with trichloroacetic acid. In the experiments with mice (Dekanski, 1945) such changes were shown not to be due to the transference of histamine from one part of the body to another, but to some physical or chemical change in the skin itself. It is probable that the changes described here are also not due to the redistribution of histamine.

It is convenient to speak of the formation or destruction of histamine, but it should be made clear that this does not necessarily imply a chemical change. The extractable histamine in the skin appears to be in equilibrium with an unknown complex of some kind. This complex may consist of a chemical system which is capable of forming and destroying the molecule, or of some substance which combines with histamine or adsorbs it and renders it inert, or insoluble in trichloroacetic acid. In the experiments with mice, similar results were obtained when the histamine was extracted by electrodialysis, so that the hypothetical insoluble substance would have to be one which was not carried through cellophane to the cathode.

If these considerations are kept in mind, the histamine in extracts can be taken as a measure of the histamine content of the skin. Possible precursors, metabolic products, and inert or insoluble compounds are not included in this term, even though they may conceivably be formed merely by the adsorption of histamine by some other substance. There is no evidence that the 'conjugated histamine' described by Anrep, Ayadi, Barsoum, Smith & Talaat (1944) is present in skin. If it were, it would be hydrolyzed during the preparation of extracts and included with the free histamine in the estimates.

The first series of experiments show that the effect of burning on the histamine content of skin depends on the temperature applied. After burning at 60° C. histamine was formed in the skin. This confirms earlier results with mice. After burning at 80° C. or 140° C. no histamine was formed, but the histamine originally present disappeared over a period of several hours. This is in agreement with results of Harris (1927). In view of these facts it seems that the formation of histamine depends on the activity of irritated living cells. Dead cells form no histamine, but on the contrary slowly lose their preformed histamine. This may be destroyed, but is probably absorbed into the general circulation.

The effect of tannic acid on the histamine content of both normal and moderately burnt skin is interesting. It seems that the powerful anti-histamine action of this substance depends upon its ability to penetrate deeply into both

the normal and burnt epidermis and even into the corium. The moderately burnt skin treated with tannic acid showed less oedema. Tannic acid or tannates in excess may be absorbed in sufficient amount to damage the liver (Cameron, Milton & Allen, 1943). This may be less dangerous when treatment is applied in a single or carefully repeated spray or by swabbing in the very early stage of injury, without scraping away epidermis.

On the other hand, silver nitrate seems to have an irritating action, encouraging oedema and the formation of histamine even in unburnt skin. There is no experimental indication for silver nitrate treatment in cutaneous burns.

The effect of gentian violet is interesting, because the histamine content of the moderately burnt skin dropped to almost normal, but the oedema persisted. It seems likely that some factor or factors other than histamine were responsible for the oedema and escaped the action of gentian violet or, alternatively, that the penetration of this dye into the skin was insufficient to prevent oedema. It was observed that formalin counteracted the increase of histamine and the appearance of oedema following burns. It seems to have relatively high penetrating power but is painful if applied to burned skin. The results obtained with 'Mercurochrome' are uncertain and cannot usefully be discussed, although it has a remarkable ability to prevent oedema.

Summary

1. Small areas of cat's skin were burned under anaesthesia and then extracted with trichloroacetic acid. After moderate burns ($60°$ C.) the histamine equivalent increased in the burned area. After severe burns ($80°$ C. and $140°$ C.) the histamine originally present slowly disappeared.

2. Tannic acid prevented the development of oedema and diminished the extractable histamine of both burned and normal skin.

3. Silver nitrate increased the extractable histamine even in unburned skin, and did not prevent oedema.

4. Gentian violet prevented the increase of histamine but not the development of oedema.

5. Formalin prevented both the increase of histamine and the development of oedema.

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THE EFFECTS OF ANALEPTICS ON
THE FATIGUED SUBJECT

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This investigation was undertaken in 1942 at the request of the Military Personnel Research Committee of the Medical Research Council. There were three main points to be studied. First, did analeptics really increase the output of work of fatigued men, and, if so, what dosage was required? Secondly, would the analeptics keep tired men awake and able to perform their duties satisfactorily? And thirdly, if the drugs did have these effects, what was the cost to the organism? That is, was the burst of energy accompanied by untoward symptoms and was it followed by a dangerous 'flop'? The results were wanted quickly so the methods adopted were of necessity the most direct possible.

Two analeptics were studied. In the earlier experiments benzedrine (β-phenylisopropylamine sulphate) was given in doses of 10–15 mg. Later, methedrine (N-methyl-β-phenylisopropylamine hydrochloride) was used, generally in 10 mg. doses. Methedrine is the same as the 'Pervitin' employed by the Germans. In all our experiments the drugs were given orally, the tablets being crushed in the mouth and washed down with water. In the control experiments, tablets of identical appearance and excipient, but containing no analeptic, were used.

The experiments fell into two main categories, laboratory studies and field studies.

I. LABORATORY STUDIES

(a) ERGOMETER EXPERIMENTS

Methods

Six men from R.A.M.C. and R.A.S.C. units acted as subjects. Their ages ranged from 22 to 28 years and they were of very varied physique. To determine whether output of work was increased by the drugs, two ergometers were used, a cycle and a hand machine. The cycle ergometer (McCall & Smellie, 1931) was set for a constant load of 2 kg. and the subject attempted to keep to a constant pedalling speed of 52 r.p.m. set by metronome. As fatigue set in, the rate of pedalling gradually fell and any changes were noted by taking readings of the total revolutions of the back wheel every 15 min. These readings were graphed against time and represented the work done per 15 min.

When the subject became exhausted and could no longer keep up even a steady slow speed, the trip on the motor controlling the magnetic brake was automatically knocked off. When this had occurred three times the experiment was stopped, a completely objective end point being thus achieved.

With the hand ergometer (Cathcart, Wishart & McCall, 1923) the procedure was practically identical, the load again being 2 kg. and the set speed 52 r.p.m. The experiment ended when the machine jammed at top dead centre on three successive occasions.

As a rule each subject worked for two successive days on the ergometers and on the third day performed various other tests such as a Static Effort Test, Flack (40 mm. Hg endurance) Test or Mosso's Ergograph Test.
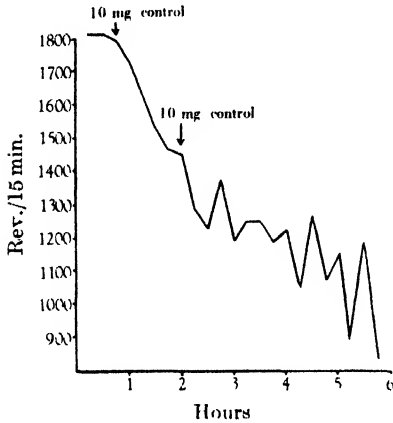
Fig. 1.

Fig. 2.

Fig. 1. Subject 2 B. Typical control experiment on hand ergometer. Ordinate, revs per 15 min of ergometer flywheel; abscissa time in hr., constant load of 2 kg.; control tablets (10 mg.) given at arrows.
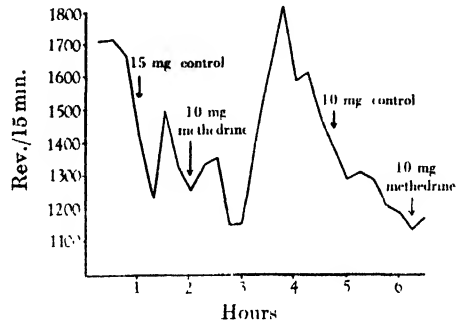
Fig. 2. Subject 2 B. Effect of 10 mg. methedrine on work output Hand ergometer, load 2 kg.; medication as indicated by arrows.
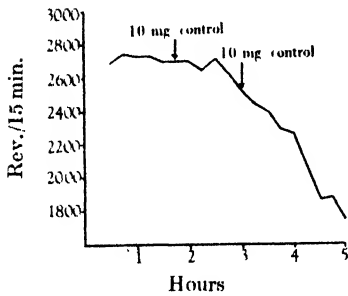
Fig. 3.

Fig. 4.

Fig. 3. Subject 2 B. Typical control experiment on cycle ergometer. Ordinate, revs per 15 min. of rear wheel; abscissa: time in hr; constant load of 2 kg.; medication as indicated at arrows.
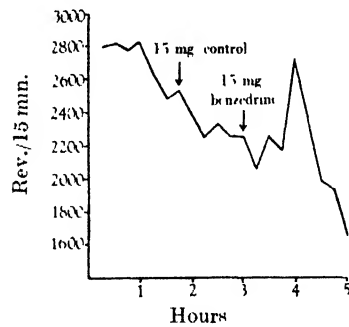
Fig. 4. Subject 2 B. Effect of 15 mg. benzedrine on work output. Cycle ergometer, load 2 kg.; medication as indicated at arrows.

Results

Fig. 1 shows the results of a typical control experiment on the hand ergometer. The graph of work done steadily falls, with minor fluctuations, the two doses of control tablets having no definite effect. Fig. 2 from the same subject on the

same ergometer shows that, when administered at the appropriate time, 10 mg. of methedrine markedly increased the output of work. Figs. 3 and 4 illustrate a similar effect, this time produced by 15 mg. of benzedrine.

Each subject, however, responded in his own characteristic way, the effect produced being fairly constant in any given subject. For example, in most
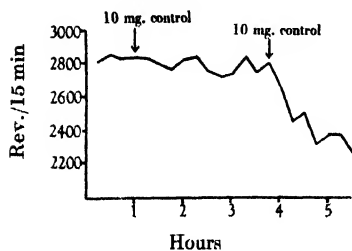
Fig. 5.
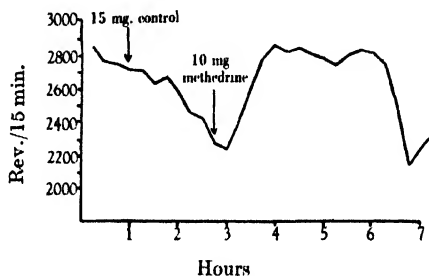
Fig. 6.

Fig. 5. Subject 1 B. Control experiment on cycle ergometer. Load 2 kg.; medication as indicated at arrows.

Fig. 6. Subject 1 B. Effect of 10 mg. methedrine on work output. Cycle ergometer, load 2 kg.; medication as indicated at arrows.
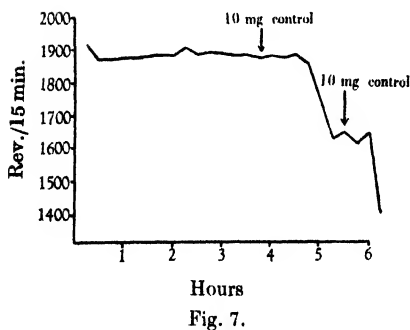
Fig. 7.
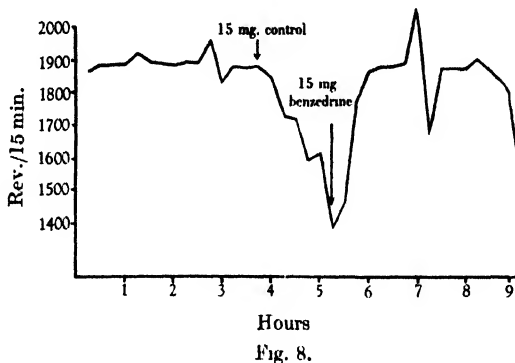
Fig. 8.

Fig. 7. Subject 1 B. Control experiment on hand ergometer. Load 2 kg.; medication as indicated at arrows.

Fig. 8. Subject 1 B. Effect of 15 mg. benzedrine on work output. Hand ergometer, load 2 kg.; medication as indicated at arrows.

cases the initial optimal rate of working was never quite achieved when the drug was given during the period of deterioration, but in one subject (1 B) the analeptic had a most dramatic and sustained effect. The effect of 10 mg. of methedrine on this subject using the cycle ergometer is seen in Figs. 5 and 6, and the effect of 15 mg. benzedrine in Figs. 7 and 8, using the hand ergometer. On another occasion this subject worked on the hand ergometer for 11¼ hr. without a break after receiving 10 mg. methedrine at 7 hr. when he was showing marked signs of failing.

Another subject (3 B) showed no response to benzedrine or methedrine given during the period of work (Figs. 9 and 10). If, however, the drug was given some hours before work commenced then the response became evident as shown in Fig. 11. The effect with this subject therefore seemed to be greatly delayed. Fig. 10 also illustrates the constancy of the results in a given subject.
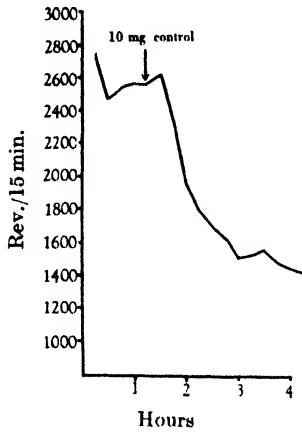
Fig. 9.
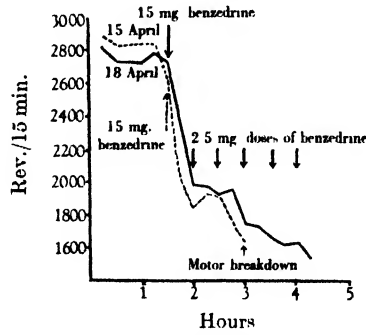
Fig. 10.

Fig. 9. Subject 3 B. Control experiment on cycle ergometer. Load 2 kg.; medication as indicated at arrow.

Fig. 10. Subject 3 B. Effect of 15 mg. benzedrine on work output. Cycle ergometer, load 2 kg., two separate experiments shown; medication as indicated at arrows.
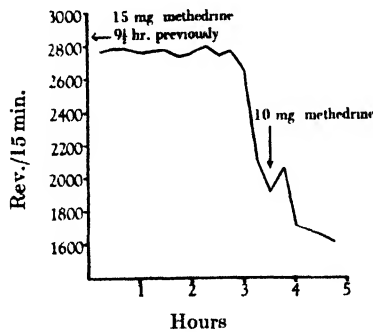
Fig. 11. Subject 3 B. Cycle ergometer, load 2 kg.; effect on work output of 15 mg. methedrine given 9½ hr. before work commenced. (Compare with Figs. 9 and 10.)

In the other subjects the stimulant action of the analeptics appeared usually within ½–1½ hr. and lasted from ¾ to 3 hr. If they were given late in the experiment no recovery was seen as the subject became exhausted before the drug took effect.

The subjective responses were not so consistent as the objective ones; but euphoria as indicated by singing, whistling and general cheerfulness was noted

in some of the subjects following the analeptics. In others, stiffness and ache disappeared temporarily and the subjects felt fresher and sometimes in their own words 'as fresh as at the start'. On some occasions the subjects complained of headache, tiredness and general 'muzziness' about 9–10 hr. after the analeptic and at times there was a history of nausea. These symptoms were, however, transient.

(b) MISCELLANEOUS TESTS

Strength tests. In most of the later experiments the hand-grip, lumbar pull and crushing power were determined before and after work on the ergometers. The greatest diminution in strength was found after the hand-ergometer experiments as was to be expected. There was, however, very little deterioration in the total strength as shown by these tests after work on the cycle ergometer, indicating that the fatigue was essentially local so far as motor activity was concerned. The analeptics had little effect on the results of these tests.

Static effort. The maximum time which the subject could hold the springs of a muscle developer at a given mark (7·5 kg. pull) with his arms at a right angle in the vertical plane, was determined at 15 min. intervals before and after the analeptics. In spite of attempts to standardize the procedure the results were so variable on account of the additional muscles which the subject could bring into play, that this type of experiment was subsequently abandoned.

Flack test (40 *mm. mercury endurance test*). This cardio-respiratory test was performed at ½ hr. intervals by three subjects before and after the administration of 10 mg. methedrine. No significant improvement in performance was noted. The manometer displacement and the heart rate were recorded graphically throughout the test.

Mosso's Ergograph. The finger ergograph did not prove a satisfactory apparatus for investigating the problem as the subjects did not exert their maximal effort after the initial stages and simply produced an abbreviated contraction which could be maintained for 4 or 5 hr. with a 7 lb. load. If maximal contractions only had been investigated the exercise period would have been so short that the analeptic would not have had time to act.

Reaction times. These were investigated by means of a motor-car chassis. The subject operated the steering, accelerator, brakes and clutch in response to light signals given every few seconds for an hour. The reaction times for each action were recorded continuously in tenths of a second by electrical means. There was no definite evidence of improvement when analeptics were given to the three subjects employed, as the effects of practice were very difficult to eliminate.

(c) CARDIOVASCULAR STUDIES

The general data on pulse rate and blood pressure obtained during the ergometer studies were first examined. Then experiments on the effect of methedrine and of control tablets on the performance of a standard cardiac tolerance test were carried out. As it was difficult to obtain reliable blood-pressure readings during the tolerance test a separate series of experiments was performed to determine the effect of methedrine on the blood pressure, pulse rate and temperature of subjects at rest. During these latter experiments the reaction of the pupils was also noted. As there is a considerable literature on the effects of benzedrine sulphate on the heart, but little on the effects of methedrine, it was decided to use only the latter drug in the cardiovascular experiments.

Methods

The cardiac tolerance test. Three subjects were employed for this test which is concerned only with the response of the heart rate to a brief standard exercise (Knox, 1940). The exercise consisted in climbing five times up and down two steps each 10 in. high, stepping in time with a metronome beating 96/min. The subject started from and returned to the sitting position. The heart beats were recorded electrically on a smoked drum before, during and after the exercise which lasted approximately 20 sec.

The heart rate was counted to the nearest tenth of a beat in intervals of 5 sec from the beginning of the exercise. The highest rate obtained in a 5 sec interval was converted to beats per min. and taken as the maximum rate. The heart rate in the 10 sec period immediately preceding the exercise, converted to beats per min., was taken as the initial rate

This test was performed at intervals of ½ hr and after the first three or four tests the subject received either 10 mg. methedrine or equivalent control tablets

Experiments on the resting subject. Eight men from R.A.M.C. and R.A.S.C. units acted as subjects. (Three of these had already taken part in the ergometer experiments.) 1½ hr. after breakfast the men were seated at separate tables and rested quietly for 15 min. when the first readings were taken. Records of pulse rate, blood pressure and size of pupils, in that order, were made thereafter at intervals of 15 min., and the temperature (under the tongue) was read every ½ hr. During the intervals, the subjects were allowed to read. After 1 hr. the methedrine or control tablet was given and the readings continued. General behaviour and signs were noted. Symptoms were elicited by questioning.

Results

Cardiac tolerance test. Previous work by one of us (J.A.C.K.) had shown that the maximum rate was probably the most reliable index of cardiac response in the tolerance test. Accordingly, the values of the initial rates and maximum rates in the successive tests at ½ hr. intervals were graphed as in Fig. 12, which shows a typical result. Prior to medication the values for maximum rate and increment over initial rate were well within the normal limits for this test, and neither the analeptic nor the control tablets caused any significant alteration in the response to the exercise. Thus a dose of methedrine (10 mg.) which caused a definite increase of work output as shown by the ergometer experiments, did not interfere with the normal cardiac acceleration as demonstrated by this test. Fisher (1937) described an instability of the pulse rate after

30–40 mg. benzedrine. In his subjects the pulse was slow at rest but the slightest exertion caused a great increase in rate. This effect was not seen in the present series, probably because of the much smaller dosage employed.
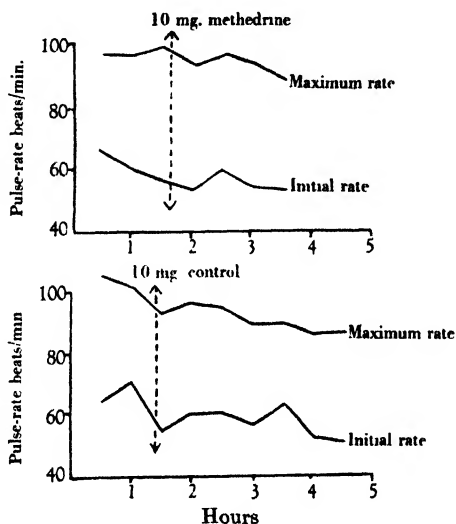
Fig. 12. Typical result of cardiac tolerance test. Graphs of initial rates and maximum rates in the successive tolerance tests performed at $\frac{1}{2}$ hr. intervals. Upper figure, effect of 10 mg. methedrine given at arrow; lower figure, effect of 10 mg. control given at arrow.

The effect of methedrine on the resting subject

Pulse rate. When the subjects received control tablets the pulse rates steadily fell during the 3–4 hr. of observation. When they were given 10 mg. methedrine this same trend was shown until about $\frac{3}{4}$ hr. after the administration of the drug; the fall was then arrested and in some cases a slight irregular rise was noted. Similar results, but with a more definite tendency towards a sustained rise, were found after 20 mg. methedrine. Previous observations by Myerson, Loman & Dameshek (1936) and Myerson (1940) indicated a slowing of the pulse rate. Davidoff & Reifenstein (1938), however, found the changes in pulse rate after 10-30 mg. benzedrine given orally to be quite unpredictable, and, recently, Finkelstein, Alpern & Gantt (1945) obtained no change in the resting rate after 20–30 mg. benzedrine.

Blood pressure. In practically all cases the systolic blood pressure (S.B.P.) tended to fall slightly before medication; after control tablets, this fall usually ceased and in some cases a rise was shown. The diastolic pressure (D.B.P.) did not usually show a marked initial fall, and after the administration of the control tablets it tended to fluctuate in parallel with the S.B.P. 10 mg. of methedrine arrested the initial fall of S.B.P. and in practically all subjects caused a slight rise of some 10–20 mm. The D.B.P. varied similarly. With 20 mg.

of methedrine six of the eight subjects showed a distinct rise in s.b.p., one subject a slight sustained rise, and one subject a fall of some 20 mm. This last man gave no definite response to 10 mg. of methedrine. Dyer (1939) has drawn attention to such an anomalous result and records a case where the s.b.p. fell by 25 mm. after 20-30 mg. benzedrine by mouth. He also noted that less than 10% of his subjects gave a significant rise in s.b.p. after 10-20 mg. benzedrine, but with a dosage of 30 mg. (approximately equivalent to our 20 mg. methedrine) the incidence of s.b.p. increase was much greater. In our subjects the d.b.p. fluctuated more or less in parallel with the s.b.p. This is contrary to Dyer's finding that the d.b.p. did not undergo significant alteration.

Temperature. The fall in body temperature of the subjects when sitting for 3 hr. in a cool room was not, in general. affected by the control tablets or by 10 mg. methedrine. With 20 mg. methedrine six of the eight subjects showed a slight rise (0·2-1° F.) commencing 1¼-2 hr. after the drug was given. Dill, Johnson & Daly (1939) stated that 20 mg. benzedrine intramuscularly increased the metabolic rate by some 7%. It is of interest that the slight thermogenic action noted in our subjects occurred after a longer latent period than the other responses, and that the rise did not in any case exceed the pre-methedrine values.

Fig. 13 shows graphs of blood pressures, pulse rate and temperature for an average subject and Fig. 14 those for the anomalous subject.

Pupils. Myerson (1940) noted pupillary dilatation after benzedrine. In our subjects, little, if any, change occurred after 10 mg. methedrine; but 30-45 min. after the administration of 20 mg. methedrine by mouth some of the subjects stated spontaneously that the lights had become brighter. Three of the eight subjects showed definite pupillary dilatation.

General behaviour and other signs and symptoms

Behaviour. No appreciable alteration in behaviour resulted from 10 mg. methedrine, but about 30-45 min. after 20 mg. methedrine the subjects became noticeably talkative and cheerful, and most of them were unable to concentrate on reading.

Symptoms following 10 mg. methedrine. The symptoms arising from this dosage were, on the whole, very mild. About 45 min. after medication one subject complained of a 'nervous feeling' and another felt 'energetic'. Two others complained of slight headache in the afternoon and early evening.

Symptoms following 20 mg. methedrine. In six of the eight subjects this dosage gave rise to a definite state of euphoria. Of the remaining two, one had visual disturbances and the other felt 'jumpy' and had a 'dullness' in his head. It is noteworthy that this latter subject was the man who showed a continuous fall in b.p. after the drug. A little later some of the subjects felt warm and clammy and some were thirsty. After about 1½ hr. the feeling of warmth
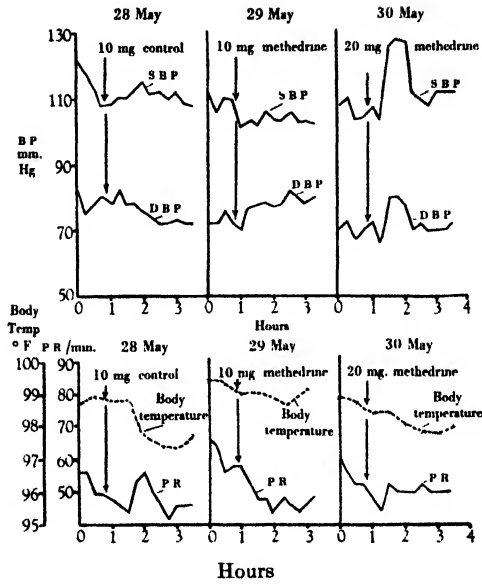
Fig. 13. Graphs showing usual effect of control tablets, 10 mg. methedrine and 20 mg. methedrine on blood pressure, pulse rate and temperature of resting subject.
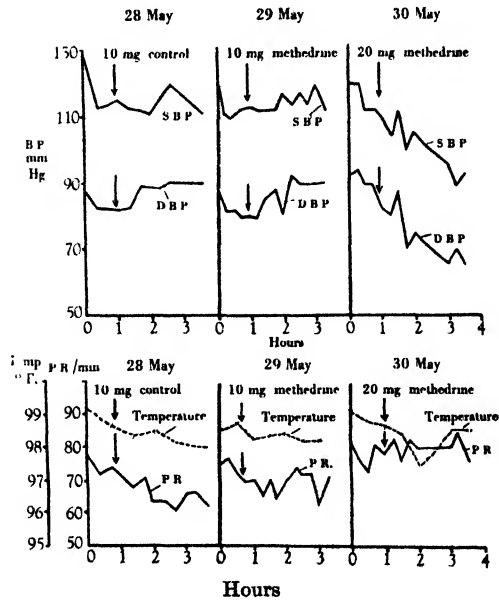
Fig. 14. Effect of control tablets, 10 mg. methedrine and 20 mg. methedrine on blood pressure, pulse rate and temperature in subject showing anomalous responses.

passed off and the subjects tended to feel cold. The euphoria was frequently succeeded by slight headache or a feeling of nervous tension. The subject who exhibited the fall in B.P. felt very drowsy about 90 min. after the drug was taken. At intervals some of the subjects experienced periods of 'jumpiness' or 'excitement' or 'strain'. One felt hungry but unable to eat, and sleepy but could not sleep. Later he became very sensitive to noises, finding them at times almost unbearable. Inability to concentrate was also a noticeable feature. With one exception, all the subjects had considerable difficulty in getting to sleep, and the quality of the sleep was also disturbed.

In only two of the eight subjects was there a return to normality 24 hr. after 20 mg. methedrine. In five of the subjects some of the most characteristic symptoms of the previous day tended to recur as they began to move about.

II. FIELD STUDIES

(a) FIRST FIELD STUDY

This was carried out to determine whether analeptics influenced marching performance and resistance to fatigue under more natural conditions.

Methods

Fifty-five officers and men of a university senior training corps were kept without sleep for 24 hr., the night being spent in performing light drills, games, gymnastics, etc., with intervals for meals and reading.

In the morning they were divided at random into three approximately equal squads and were sent out on an 18 miles route march. The squads were separated by $\frac{1}{2}$ hr. intervals so that they did not come within sight of each other during the march. Before setting off, the members of the leading squad (squad X) received 15 mg. methedrine, those of the rear squad (squad Z) were given control tablets, while the middle squad (squad Y) was a mixed group, half receiving 15 mg. methedrine and half the control tablets. The subjects were, of course, unaware of the nature of the tablets they received. Every 2 hr. during the march a 10 min. halt was made, during which drinking water was supplied but no food was allowed. A cinema record of the marching was made at intervals unknown to the cadets. Foot inspections were held before and after the march. Reports were filled in by all ranks after the march and on the following morning.

Results

The main results are summarized in Table 1, the various items of which are discussed briefly below.

1. Neither the total time taken to cover the course nor the cinema records of the style of marching showed any definite difference between the groups.

2 and 3. The total number of men complaining of foot trouble was not significantly different in the two groups but *complaints* of severe foot trouble were commoner in the control group. On the other hand, examination of the feet after the march showed that whereas only 11 men in the control group had blistered feet, 18 men in the methedrinized group were affected, and moreover the *number* of severe blisters was also greater in this group. The evidence thus

indicated that the methedrinized men, though suffering to a greater extent from blistered feet, complained less than the controls.

4. The total number of men who fell out on the march for various reasons was definitely greater in the control group than in the methedrinized group and the importance of this observation is enhanced by the fact that foot trouble was more marked in the drugged group.

5. Although the total number of men complaining of fatigue after the march was practically the same in the two groups, the number complaining only of slight fatigue was greater in the methedrine group. There was thus some evidence that, despite their more severely blistered feet, they felt the effects of fatigue less than the controls.

6. To the question 'Could you have gone further?' twice as many affirmatives came from the methedrine group as from the controls.

7, 8 and 9. Subjectively, methedrine definitely appeared to help the men whereas the control tablets did not have this effect to anything like the same extent. Further, euphoria was experienced by 11 men in the methedrine group

TABLE 1. Summary of results from first field study

		Methedrine group			Control group		
Item no.	Datum	Squad X 18 men	Squad Y 9 men	Total 27 men	Squad Y 10 men	Squad Z 18 men	Total 28 men
1	Time taken to cover 18 miles (including halts)	5 hr. 24 min.	5 hr. 22 min.	—	5 hr. 22 min.	5 hr. 28 min.	—
2	Trouble with feet during march (subjective):						
	None	3	4	7	4	5	9
	Slight	7	1	8	1	4	5
	Marked	5	3	8	2	3	5
	Severe	2	1	3	1	6	7
	Total affected	14	5	19	4	13	17
3	Blistered feet after march:						
	Slight	5	1	6	1	1	2
	Moderate	2	3	5	2	3	5
	Severe	6	1	7	2	2	4
	Total men affected	13	5	18	5	6	11
4	Number who fell out during march	3	0	3	2	5	7
5	Degree of fatigue after the march:						
	Slight	8	4	12	3	2	5
	Moderate	4	1	5	2	7	9
	Severe	5	4	9	2	8	10
	Total fatigued	17	9	26	7	17	24
6	Number who said they would have been able to march for longer period	8	3	11	2	3	5
	Number who said they could not	7	3	10	2	14	16

TABLE 1 (*cont.*)

Item no.	Datum	Methedrine group			Control group		
		Squad X 18 men	Squad Y 9 men	Total 27 men	Squad Y 10 men	Squad Z 18 men	Total 28 men
7	Number who thought drug or control helped during march:						
	Yes	13	5	18	2	3	5
	No	5	3	8	4	6	10
8	Number who thought drug or control caused unusual symptoms apart from euphoria:						
	Yes	10	0	10	1	4	5
	No	5	4	9	3	14	17
9	Euphoria during march:						
	Yes	10	1	11	0	1	1
	No	8	8	16	10	17	27
10	Degree of thirst during march:						
	Slight	4	4	8	5	11	16
	Moderate	6	2	8	0	3	3
	Marked	4	0	4	1	1	2
	Total affected	14	6	20	6	15	21
11	Degree of hunger during march·						
	Slight	2	1	3	4	5	9
	Moderate	0	0	0	0	1	1
	Marked	0	0	0	0	1	1
	Total affected	2	1	3	4	7	11
12	Unusual symptoms on night following march:						
	Headache	3	1	4	1	0	1
	Dilated pupils	5	0	5	0	0	0
13	Character of sleep on night following march:						
	Good	13	6	19	8	13	21
	Moderate	4	2	6	0	1	1
	Bad	0	0	0	0	1	1
	*Unknown	1	1	2	2	3	5
14	Mean time of going to bed (24 hr. clock)	2300	2130		2045	2045	
15	Mean time slept	10 hr. —	11 hr. 30 min.	10 hr. 30 min.	13 hr. –	12 hr. 24 min.	12 hr. 36 min.
16	General condition on following morning:						
	Fresh	4	3	7	4	5	9
	Pleasantly tired	9	3	12	1	6	7
	Moderately tired	3	2	5	2	2	4
	Fatigued	1	0	1	1	0	1
	*Unknown	1	1	2	2	5	7

17 O'Grady drills

	% of total errors made by each squad	
	Squad X	Squad Z
01.00 hr. drill A (½ hr.)	65	35
08.40 hr. drill B (¼ hr.)	61	39
15.33 hr. drill C (¼ hr.)	68	32

* Satisfactory replies were not obtained from these subjects.

and by only one man in the control group. The symptoms were generally slight. One subject, who had received methedrine, became, however, completely disoriented for a time. He staggered along in a dazed sort of way, but managed to keep up with the others and later became quite normal again and duly finished the march. Another incident was of great interest from the military point of view. The N.C.O. in charge of the methedrine squad, an experienced soldier and usually a very reliable man, made a gross error of judgement. About 1 hr. after receiving the analeptic he was very anxious that his squad be allowed to take an alternative route which would have added an extra 10 miles to the march, as he was sure the men could manage it easily. Later, when he had recovered from the drug, he said that he was thankful that he had not been allowed to have his way, as the men would obviously have foundered. It is to be noted that the cadets were medical students, in no special training.

10 and 11. Though drinking water was supplied on the route, thirst rather than hunger was noticed in the methedrine group and the reverse in the control group.

12. It is of some interest that a few men in the methedrine group complained of headache, and five out of the twenty-seven had dilated pupils.

13, 14, 15 and 16. The sleep rhythm on the night following the march was but little affected by the methedrine taken the same morning. On the average, the control group went to bed earlier than the methedrine group. A noteworthy feature of the experiment was the complete absence of 'flop' or even of severe 'hang-over' after the effects of the drug had worn off.

17. In an attempt to assess the alertness of the men, 'O'Grady drills' were performed twice during the night preceding the march and once on returning from the march. ('O'Grady drill' consists of a series of commands which may or may not be prefaced by the words 'O'Grady says--'. Only those commands prefaced should be obeyed.) Although there was a definite original intrinsic difference in the number of errors made by the X and Z squads, this difference was not significantly altered after medication. The general conclusion from this study was that 15 mg. of methedrine did diminish the fatigue and discomfort of a long route march, but that certain unfavourable symptoms might occur.

(b) SECOND FIELD STUDY

The purpose of this study was to determine whether benzedrine in 10–15 mg. doses would keep physically tired men awake.

Method

Two companies of a fully trained infantry battalion acted as subjects. They were employed on a 48 hr. operations scheme as shown in Table 2. One company was given benzedrine as indicated, one-quarter of its personnel receiving 15 mg. and the rest 10 mg. The other company was given identical numbers of control tablets at the same times. The officers and men filled in a questionnaire relating to each 24 hr. period and this supplemented the reports of the observers.

TABLE 2. Second field study. Time-table

First day

0715–1600 hr.   March of 23 miles, with halts
1900   hr.   Tablets given (10–15 mg. benzedrine or control)
2200–0300 hr.   Sleep in the open

Second day

0300–1700 hr.   March of 20 miles and skirmish with enemy
1800   hr.   Tablets given (10–15 mg. benzedrine or control)
2230–0730 hr.   River crossing and attack

Results

When the men lay down to sleep in the open after a march of 23 miles, it was found that 10 mg. of benzedrine, given 3 hr. before, disturbed their sleep, tending to change a moderate sleep into a bad one. With 15 mg. benzedrine under similar conditions the effect was greater, 64% of the men receiving this dosage could not sleep, compared with only 4% in the control group. The effects on the quality of sleep are summarized in Fig. 15. In spite of the poor quality or absence of sleep the benzedrine group rose in the morning feeling less fatigued than the controls, and this benefit was still apparent at the end of the 48 hr. period. There was some evidence that the effects of the drug wore off more rapidly after the second administration. Very approximately, the activity seemed to last for some 12–16 hr. after the first dose and some 5–8 hr. after the second. The men were, of course, becoming more exhausted towards the close of the operation. Following both periods of administration of the drug there was no more evidence of 'hangover' than there was in the control group.

Fig. 15. Frequency distribution of quality of sleep in men receiving control tablets, 10 mg. benzedrine or 15 mg. benzedrine. (Second field study.)

From the questionnaire it was found that 70% of the men who received benzedrine thought that the tablets did diminish discomfort and fatigue, whereas only 23% of the men in the control group thought that their tablets had this effect. There was also some evidence that, as in the first field study, thirst was more apparent in the group receiving the analeptic.

It was therefore shown that 10–15 mg. of benzedrine did produce increased wakefulness which undoubtedly might be of value in certain emergencies.

Discussion

Lehmann, Straub & Szakáll (1939) found that doses of 5 or 10 mg. methedrine increased the performance on the bicycle ergometer in all three of their subjects. They attributed this result to central excitation which reduced the protective mechanisms such as fatigue, muscle pain, etc., which normally guard against over-exhaustion. Knoefel (1943) obtained similar increases in work output with 10 mg. methedrine in five out of seven subjects on the cycle ergometer. Alles & Feigen (1942), using Mosso's ergograph, found that 10, 20 or 40 mg. of benzedrine inhibited the onset of voluntary muscle fatigue and stated that the effect was probably primarily due to an action directly on the central nervous system. This increase in work output was confirmed by our ergometer experiments, but we would stress the individual variations which occurred. In a given subject, however, the effects could be predicted fairly accurately after a few trials. In the work experiments the effect usually appeared within $\frac{1}{2}$–$1\frac{1}{2}$ hr. and lasted from $\frac{3}{4}$ to 3 hr. The time taken to act is in general agreement with the $\frac{3}{4}$–2 hr. stated by Peoples & Guttmann (1936), and the results of Beyer (1939) and Alles & Feigen (1942). In the ergometer experiments our subjects received dosages of the analeptics up to the equivalent of 163 mg. benzedrine spread over 16 days, and this raised the question whether continued administration of these drugs has a deleterious effect. This has been reviewed in a recent article by Shorvon (1945) in which relatively enormous doses of benzedrine, up to 125 mg. daily over a long period, were found to produce no evidence of physiological damage. Apart from some disturbance of sleep when the analeptics were given in the evening, our subjects showed no definite evidence of unfavourable sequelae.

In general, it was found that 10 mg. of methedrine were approximately equivalent to 15 mg. of benzedrine, and this is in agreement with the results of Larsen (1938) and Jacobsen, Christensen, Eriksen & Hald (1938).

When analeptics are employed to sustain work output or to diminish fatigue one of the main limiting factors would appear to be the development of untoward effects such as irrational behaviour, visual disturbances and depression. Thus Carl & Turner (1940) found that small doses of benzedrine increased psychomotor output without affecting accuracy whereas larger doses caused confusion. It is therefore necessary to balance the beneficial effects, such as increased output of work and alertness, against the unfavourable effects. It may be useful to summarize our findings concerning those effects in relation to the dosage of methedrine, which is approximately $1\frac{1}{2}$ times as potent as benzedrine. Apart from the occasional development of slight headache, muzziness and nausea, 10 mg. methedrine would seem to be a safe and effective dose under the conditions described in the present paper. The effects of 15 mg. methedrine were observed in the first field study. Here, though on the whole

the drug was probably beneficial, the deleterious effects were undoubtedly more marked, and included thirst, headache, and in one case at least, definite irrationality. The general symptomatic effects of 20 mg. methedrine were seen in the cardiovascular studies. It was obvious that the greater dosage caused an increase in untoward symptoms. Apart from the doubtful effect of the temporary euphoria all other sequelae were such as might well diminish the general efficiency. Under certain circumstances even the euphoria might prove a disadvantage as it tends to cause a certain amount of restlessness, lack of concentration and noisiness. A curious feature of the symptomatology with this dosage was the tendency to periodic recurrence of the main symptoms.

Under the conditions of the ergometer experiments in which the drug was given when deterioration in performance was either imminent or evident, the action appeared to be exerted over a relatively short period. In the cardiovascular and field studies where an increased dose was given, the onset and duration of the euphoria corresponded fairly well to that of the increased work output in the ergometer studies. The deleterious effects, however, following the increased dosage, gradually became evident as the euphoria declined and lasted with varying intensity for about 24 hr. We are, therefore, of the opinion that 20 mg. methedrine given at one time is an overdose for most subjects.

With regard to the possible uses of analeptics in war it seems clear that these drugs should not be given indiscriminately to large numbers of men because of the wide individual variations in response. Euphoria, noisiness or lack of judgement in even a few sensitive individuals might well upset an entire operation. On the other hand, it is probable that the use of analeptics in emergencies by individuals or by small groups of men whose reactions to the drug are already known, may sometimes be justified.

Summary

1. Oral administration of 15 mg. benzedrine or of 10 mg. methedrine increased the subject's capacity to sustain a given level of work performance on ergometers. If deterioration had already set in, these analeptics caused the work output to rise within $\frac{1}{2}$-$1\frac{1}{2}$ hr. and maintained the increased rate of working for generally about 1 hr. There was usually subjective improvement corresponding to the objective response. Methedrine was found to be about $1\frac{1}{2}$ times as potent as benzedrine.

2. There were wide individual variations in response to the drugs but in a given subject the effects could be predicted fairly accurately after a few trials.

3. The effects of 10 and 20 mg. methedrine on the blood pressure, pulse rate, temperature and general behaviour of resting subjects are described.

4. 15 mg. methedrine diminished the fatigue and discomfort of a long route march, but certain unfavourable signs and symptoms occurred.

5. 10–15 mg. benzedrine caused increased wakefulness in fatigued men under the conditions of a military exercise.

6. Moderate doses of these analeptics were not followed by any marked 'flop'.

7. 10 mg. methedrine, or 15 mg. benzedrine, was found to be a safe and effective dose under the conditions described, but 20 mg. methedrine, or 30 mg. benzedrine, was an overdose for most subjects.

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BINOCULAR SUMMATION WITHIN THE NERVOUS PATHWAYS OF THE PUPILLARY LIGHT REFLEX

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The question 'Does simultaneous stimulation of the retina of both eyes give rise to a greater response than stimulation of one eye only?' has interested investigators since the last century. Lythgoe & Phillips (1937), who give references to previous authors, measured the change in light threshold during dark adaptation for right, left and both eyes and found that the binocular threshold was lower than the monocular. In both foveal and peripheral vision the binocular threshold was approximately the same as the monocular threshold for a test patch of twice the area, a finding which showed that a moderate degree of summation had occurred when both eyes had been used. Crawford (1940) has repeated the work of Lythgoe & Phillips with substantially similar results and, in addition, has measured the minimum detectable difference of brightness at various brightness levels for both eyes and for a single eye. These measurements showed only a small amount of summation at all brightness levels when the central fovea was used. With the parafovea (8°) two eyes were about 30% more sensitive than one eye to differences of brightness, except at low brightness levels when only a small amount of summation was obtained. Clearly the brightness threshold behaves in a different manner from the light threshold. Crawford gives no explanation of this difference. Pirenne (1943), using a new method of light threshold measurement, has found that there is a small degree of summation in the peripheral retina (20°). He presented several series of 4 m./sec. flashes of light to the eye and for each series determined the number of flashes seen by the observer. The 'frequency of seeing' with both eyes was somewhat greater than that obtained with one eye only, but doubling the intensity of the flashes and observing with a single eye produced a much larger increase in the 'frequency of seeing', and he concluded that under the conditions of his experiment binocular summation was small in amount. Thus it seems that the degree of summation of afferent impulses derived from two eyes depends upon the way in which the effect of these afferent impulses is to be measured. The object of the present experiments was to find out if summation of afferent impulses occurs when a purely objective effect, such as contraction of the pupil, is measured. Since the nervous pathways of the pupillary light reflex do not ascend higher than the mid-brain, the results might help to localize the site of summation within the nervous system.

METHOD

The diameter of the left pupil was measured from photographs of the eye taken with a flash of light of short duration. One or both eyes was exposed to a stimulating flash of known area and intensity and the effect of this flash upon the diameter of the left pupil was determined. The time interval between the stimulating flash and the photographic flash was approximately 0·7 sec. In a preliminary experiment with subject J.P.B.S. it was found that the pupillary response following the stimulating flash was maximal in about 1·5 sec. and thus by using a time interval of 0·7 sec. comparisons could be made during the contraction phase of the response. The diameter of the resting pupil in both subjects was about 7·2 mm. and after 0·7 sec. was about 6·2 mm.

The Ciné-Kodak Special camera, the timing apparatus and a photoflood lamp for taking the flash photographs were all mounted upon a 'gate' which was clamped in front of the subject's chair. The position of the subject's head relative to the camera could be fixed by his biting upon a dental impression mouthpiece attached to the 'gate'. The white stimulating flash was produced by even illumination of the aperture of an iris diaphragm placed at 104 cm. from the eye. The brightness of this aperture was the same for each eye and the flash (0·04 sec. approx.) was produced by a make-and-break switch in the lamp circuit. A small red fixation light was provided, so that the stimulating flash could be viewed either at the central fovea or displaced 8° horizontally into the right visual field. The image of the stimulating flash in the 8° position did not fall upon the blind spot of the right eye. The photographic flash was produced by operating an electromagnetic shutter fitted to the front of a light-tight box containing the photoflood, and this flash had a brightness of 24·6 candles/sq.cm., a duration of 0·04 sec. approx. and an area of 11·68 sq.cm. The image of the illuminated area fell upon the extreme periphery of the retina.

Before any series of experiments, a 15 min. period was allowed for the eye to become dark-adapted. The head was fixed and whilst the subject watched the fixation spot, the camera motor, which was set to take eight pictures a second, was operated and a run completed as follows. The first turn of the camera mechanism (one picture) operated the contact which provided the stimulating flash. During the next five turns the eye was in darkness; on the seventh turn a flash photograph of the left eye recorded the state of the pupil at a time approx. 0·7 sec. later than the end of the stimulating flash. About 0·5 min. sufficed to reset the apparatus and during this time the eye was resting in darkness. A further run was then made.

An experiment was undertaken to determine the effect of the photographic flash upon the resting size of the pupil. Photographs were taken either 0·5 min. or 3 min. after a preliminary photographic flash, and it was found that the pupil diameter was significantly larger at the end of the 3 min. rest than it was after 0·5 min. rest. (Mean diameter of ten measurements of pupil diameter after 3 min. rest, 7·41 mm.; mean diameter after 0·5 min. rest, 7·28 mm.; difference, 0·130 mm.±0·040 mm. standard error.) The rest period of 0·5 min. was chosen because a longer period would have made the experiments very laborious from the subject's point of view, and the failure of the pupil completely to regain the rest position in this time does not destroy the validity of the findings because each run was performed in random order, and therefore each preceding rest period was of random duration, resulting in a random resting pupil size.

At the end of each series of runs a millimetre scale was placed at the focus of the camera system and photographed. Measurements of the pupil diameter were made by projecting the image of this scale photograph on to 0·5 in. graph paper so that 1 mm. equalled 0·5 in. and then by projecting each pupil photograph through the same optical system, this 0·5 in. paper was used to measure the horizontal diameter to the nearest 0·1 mm. The diameter measured was therefore the apparent diameter as seen through the cornea and not the actual diameter.

RESULTS

Preliminary experiments showed that the method was somewhat insensitive. The results in Table 1 show the relationship between the mean pupil diameter and the area of the stimulating flash viewed monocularly. The brightness of

the flash was 0·034 candle/sq.cm. A change of area of the stimulating flash of seventy times resulted in a change of only 0·64 mm. pupil diameter, and, if the use of both eyes were equivalent to doubling the area of the stimulating flash and using one eye, then it is clear that to increase precision a large number of runs would be required, with the results treated statistically.

TABLE 1. The relationship between the pupil diameter and the area of the stimulating flash observed with one eye only

Area of stimu-lating flash (sq.cm.)	Pupil diameter (mm.)			Mean (mm.)
28·1	6·2	5·8	5·8	5·93
17·8	6·0	5·8	5·9	5 90
13·8	6·5	6·0	6 0	6·17
8·0	6·2	6·4	6·0	6·20
3·7	6·2	6·2	6·2	6·20
0 4	6·6	6·5	6·6	6·57

Accordingly, the experiments were built on the following plan. A series of triplets was performed. (i) A run using the standard area viewed with one eye. (ii) (*a*) Double, or (*b*) four times the area viewed with one eye. (iii) The same standard area as in (i) viewed with two eyes. Within the triplet the runs were presented to the observer in random order. From each triplet three pupil diameters were obtained and that for treatment (i) was compared with that for (ii) or (iii). The diameter of each (ii) or (iii) was subtracted from the corresponding (i) and thus from each series of triplets a number of differences was available for each of the two comparisons. If, in fact, runs (i) and (ii) and runs (i) and (iii) produced the same result, the mean of these differences would on the average be zero. A series of twenty-seven triplets gave the differences shown in Table 2 for the (i)–(iii) comparison. The mean difference is +0·185 mm.

TABLE 2. Twenty-seven differences of pupil diameter (mm.) obtained by comparing the figures for monocular and binocular tests

0·0	− 0·6	− 0·1
+ 0·3	+ 0·4	+ 0 2
+ 0·1	+ 0·4	+ 0·3
+ 0·4	0·0	+ 0·8
− 0·1	0·0	+ 0·1
+ 0·4	+ 0·5	+ 0·4
+ 0·1	+ 0·1	+ 0·2
0·0	+ 0·1	0·0
0·0	+ 0·8	+ 0·2

with a standard error of ± 0·055 mm. This mean difference could have arisen either as the result of random experimental variations or as a significant difference of effect between treatments (i) and (iii). To decide between these two possibilities 'Student's' distribution of *t* was used and in this case it was found that a mean difference as large, or larger, than this would on the average occur by chance in one out of every 500 series of twenty-seven differences and it was therefore extremely unlikely that this particular set of twenty-seven differences arose as the result of random experimental variation. Thus in this

case the mean difference $+0.185$ mm. indicates a significant difference of effect between the monocular and binocular runs.

The standard error ± 0.055 mm. includes such errors as those due to variations in the duration of the stimulating flash as well as those due to the variations in the response of the nervous pathways and although no precise evaluation of the relative magnitude of these errors was made, the instrumental and measuring errors appeared insignificant beside the variation in the response of the nervous pathways. The value of this experimental design is that the significance of the result can be judged in the face of both instrumental and biological errors, because the standard error, as here calculated, is a measure of the total experimental error.

Table 3 shows the results for two subjects for the comparison between monocular and binocular runs. Several series of triplets have been attempted and if 1 in 20 is taken as the level of significance, all except one series clearly show binocular summation. The stimulating flash had a brightness of 0.0060 candle/sq.cm. for the parafovea and 0.0053 candle/sq.cm. for the central fovea. It subtended an angle of $39.6'$ at the eye for the standard area. No direct comparison between the degree of summation at the central fovea and that at the parafovea was made, but from the figures in Table 3 it seems unlikely that the difference of behaviour is great.

Table 4 gives the relationship between the mean monocular-binocular difference and the mean difference between runs in which the standard area (visual angle $39.6'$) and runs in which twice ($56'$) and four times ($1°\ 19'$) the area were used. Mean differences in any one line are comparable because the differences from which they have been calculated were obtained from the same series of triplets, but owing to day-to-day variation one line cannot be compared with another in the same certainty.

The difference between the two mean differences in any one line is given in column Δ with the appropriate standard error in column σ_Δ. 'Student's' distribution of t was used to test whether or not each Δ could be regarded as significant and this for each Δ (level 1 in 20) is recorded in the final column of the table. The probability in lines 1 and 2 was obtained by graphical interpolation in the table of t and since the Δ in line 1 only just failed to reach significance and was in the same sense as that in line 2, a further test was undertaken which treated lines 1 and 2 as a whole. The χ^2 distribution was used and it was determined whether two probabilities as low as those in lines 1 and 2 would be likely to occur by chance in two tests of significance of the same phenomenon. χ^2 was 13.87 for four degrees of freedom and this value is only equalled or exceeded by chance in less than one case in a hundred, so that the Δ's in lines 1 and 2 are clearly significant if considered together.

Thus for the parafovea of subject T.E. the increase of constriction obtained with binocular instead of monocular vision was greater than the increase

TABLE 3. The relationship between the mean pupil diameters found in monocular and binocular tests

			Central fovea						Parafovea (8°)		
Date	Subject	No. of diff.	Mean diff. (mm.)	σ_{mean}	Probability	Date	Subject	No. of diff.	Mean diff. (mm.)	σ_{mean}	Probability
1. vi. 46	T.E.	25	+0·160	±0·029	<0·001	31. v. 46	T.E.	25	−0·052	±0·037	<0·20
						27. vi. 46	T.E.	25	+0·320	±0·049	<0·001
						27. vi. 46	T.P.	25	+0·236	±0·033	<0·001
4. vi. 46	J.P.B.S.	20	+0·320	±0·048	<0·001	25. iv. 46	J.P.B.S.	27	−0·185	±0·055	<0·01
						1. vi. 46	J.P.B.S.	18	+0·228	±0·087	<0·02

TABLE 4. The relationship between the increase of construction of the pupil obtained when two eyes were used instead of one, and the increase found when the area of the stimulating flash was increased whilst observing with a single eye

(All figures in this table have been approximated to three places of decimals.)

			Monocular area ×2			Binocular					
	Date	Subject	No. of diff.	Mean diff. (mm.)	σ_{mean}	No. of diff.	Mean diff. (mm.)	σ_{mean}	Λ	σ_{Λ}	Probability	Significance
(1)	27. vi. 46	T.E. (8°)	25	−0·196	±0·041	25	+0·320	±0·049	0·124	±0·064	0·059	No
(2)	27. vi. 46	T.E. (8°)	25	+0·100	±0·044	25	+0·236	±0·033	0·136	±0·055	0·017	Yes
(3)	25. iv. 46	J.P.B.S. (8°)	23	+0·009	±0·040	27	−0·185	±0·055	0·176	±0·067	<0·020	Yes

			Area ×4			Binocular					
(4)	31. v. 46	T.E. (8°)	25	+0·160	±0·048	25	+0·052	±0·037	0·108	±0·060	<0·100	No
(5)	1. vi. 46	T.E. (fovea)	24	+0·258	±0·033	25	+0·160	±0·029	0·098	±0·046	<0·050	Yes
(6)	4. vi. 46	J.P.B.S. (fovea)	20	+0·290	±0·051	20	+0·320	±0·048	0·038	±0·070	<0·700	No

obtained by doubling the area and observing with a single eye and this was also true for the parafovea of J.P.B.S. (line 3). In the parafovea of T.E. the binocular increase might be as great as that produced by increasing the area four times (line 4), but this was not so when this subject used the central fovea (line 5). In the central fovea of J.P.B.S. (line 6), on the other hand, the degree of summation corresponds well with an areal increase of four times in the single eye.

Owing to the considerable repetition of the runs, the subjects could find out from the small noises made in resetting the apparatus which type of stimulating flash they were about to see, and this knowledge might have influenced the result. To test this possible cerebral interference, a further experiment with subject J.P.B.S. was made using the parafovea. Precautions were taken to prevent the subject from finding out which flash he would see next and the success of these precautions was tested by asking him to say, just before taking a bite, which flash he thought would be the next. Runs were of four kinds, the standard area being used throughout. (*a*) Viewed with one eye and unknown to the subject. (*b*) Viewed with two eyes and unknown. (*c*) Viewed with one eye and known. (*d*) Viewed with two eyes and known. In the 'known' runs the subject was told which type of flash he would see. All four types were performed in random order. Out of the thirty-six runs in types (*a*) and (*b*) the subject declared correctly nineteen times and incorrectly seventeen times and since the chance was 1 in 2, the precautions taken to ensure that the runs were 'unknown' were satisfactory. Binocular summation was again found when runs (*a*) and (*b*) were compared ($+0.228$ mm. ± 0.087 mm.) but a significant result was not obtained with runs (*c*) and (*d*) ($+0.106$ mm. ± 0.104 mm.). It seems that if the attention of the subject is definitely directed to the type of run to be performed, a somewhat larger error will result and since an error as large as ± 0.104 mm. had not previously been found, cerebral activity is unlikely to have influenced the results in the tables.

DISCUSSION

The afferent neurons of the light reflex run from the retina to the pretectal nucleus in the mid-brain where synapses are formed with short connector neurons, themselves forming synapses with the neurons of the Edinger-Westphal portion of the oculomotor nucleus. From here the motor fibres pass via the oculomotor nerve to the constrictor muscle of the iris. Since these pathways do not utilize co-ordinating neurons above the level of the mid-brain, the binocular and areal summation found in these experiments probably occurs at the synapses within either the pretectal or oculomotor nucleus. It is thus possible that the binocular summation found with dark-adaptation threshold measurements occurs at a level below that of the cerebral cortex, perhaps in the lateral geniculate body.

SUMMARY

1. It has been shown that the degree of constriction of the pupil which results from the stimulation by light of the retina of a single eye was significantly less than that obtained when both eyes were stimulated.

2. This binocular summation was equivalent to that obtained by increasing the area of the stimulating flash between two and four times and observing with a single eye throughout.

3. This binocular summation does not appear to be influenced by cerebral cortical activity and the position of the summation within the nervous pathways is discussed.

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SUBTHRESHOLD POTENTIALS
IN MEDULLATED NERVE

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The mode by which impulses are generated in medullated axons has been the subject of much controversy. There are two conflicting views: (*a*) According to the schools of Erlanger and Kato (see e.g. Blair, 1938; Tasaki, 1940) the impulse in a normal medullated nerve fibre starts at the point of stimulation in an all-or-none fashion, and a subthreshold electric stimulus produces only local changes which are directly proportional to the applied current intensity. (*b*) Opposed to this is the view that the nerve membrane can be partially excited, and that a weak non-conducted response is elicited by subliminal electric stimuli (Rushton, 1937; Katz, 1937, 1939 *a*, *b*).

The supporters of the first view claim that there is no sign of a local action potential, nor of any non-linear reaction of the nerve to applied stimuli, until the stimulus is strong enough to elicit a transmitted response. The supporters of the second view have presented evidence which indicates that the excitatory process at the cathode increases in a progressive, non-linear fashion as threshold is approached.

The state of the controversy was reviewed by Katz (1939 *b*). The evidence for a non-conducted, graded, response was complete in the case of non-medullated nerve (Hodgkin, 1938), but not of medullated nerve, where the direct demonstration of a local action potential had not been possible. It is true that with an integrating sensitive galvanometer and high-frequency alternating stimuli, a local negative potential, of non-linear characteristic, was observed, but its time course was not known. It might conceivably have been a slow permanent depolarization rather than a summation of brief changes as required by the excitability measurements (Katz, 1937).

A further attempt has been made to throw light on this problem, with a method based on the following observation. If a brief shock was applied to a medullated nerve trunk, through two electrodes only 1–2 mm. apart, there was evidence indicating that a relatively large local response was set up at the cathode which spread to either side and soon overpowered the anelectrotonic

effect at the other stimulating lead (Katz, 1937, 1939a). From an analysis of excitability curves on the frog's sciatic nerve (Katz, 1937, Fig. 11) a brief local response was predicted, its duration at room temperature being a little over 0·5 msec. If a recording lead were placed half-way between these stimulating leads, one might hope to balance the applied potential and the electrotonic polarization effect sufficiently to detect a small local action potential.

METHODS

Most experiments were made on sciatic nerves of English *Rana temporaria* using a stretch of nerve, proximal to its main division, which is practically free of branches. In some experiments, peroneal or phalangeal nerves were used. The nerves were soaked for ½–1 hr. in oxygenated Ringer's solution and then mounted in a moist paraffin-wax chamber on adjustable platinum electrodes. In some experiments, non-polarizable electrodes were used (Ag/AgCl connected with the nerve by a 1% Agar/Ringer tube and cotton-wick). The thin phalangeal nerves were set up in paraffin oil to prevent drying.

Stimulating and recording electrodes were arranged as shown in Fig. 1. All electrodes were adjustable, the position of lead C being specially controlled by a fine screw drive, which was used to obtain optimum electric balance.

The stimulating and recording apparatus were the same as described by Katz & Schmitt (1940). The stimulus consisted of a thyratron controlled condenser shock (0·05 μF discharging through a resistance pad of about 70 Ω). The current strength was varied by two resistance pads (a coarse and fine control), the output of which was calibrated with a galvanometer. The shock was applied to the nerve via a shielded air-cored transformer (making the stimulus diphasic) and a reversing switch (Fig. 1). Single or low-frequency shocks (2–3/sec.) were used and the local potential changes recorded by a balanced resistance-capacity coupled amplifier and cathode-ray oscillograph, previously described. The oscillograph deflexion due to a rectangular input voltage, applied through sciatic nerve and platinum electrodes, reached 50% of its maximum in 12 μsec. and 90% in 35 μsec. In Fig. 1, a balanced input

Fig. 1. Arrangement of stimulating and recording electrodes. (a) A, C, E, recording leads; B, D, stimulating leads. All electrodes are adjustable, C being provided with a fine control. Distances: AC and CE about 15 mm. each; BD, 1–2 mm.; T, secondary transformer coil; S, reversing switch. (b) Equivalent bridge circuit (neglecting reactances).

is shown, and the earth applied to a centre-tapped potentiometer across the stimulating leads; this, however, was not essential, the result being unchanged if recording lead C was earthed.

RESULTS

If a weak shock, e.g. 0·2 threshold, is applied and electrode C adjusted to give the least shock artifact, a small residual potential change is obtained, which is due to an unbalanced remainder of stimulus and electrotonic potential. It has a very rapid initial component, due to the brief applied potential (which is distorted by the stray capacities of the recording apparatus), followed by a slower and smaller potential change which is largely due to polarization in the

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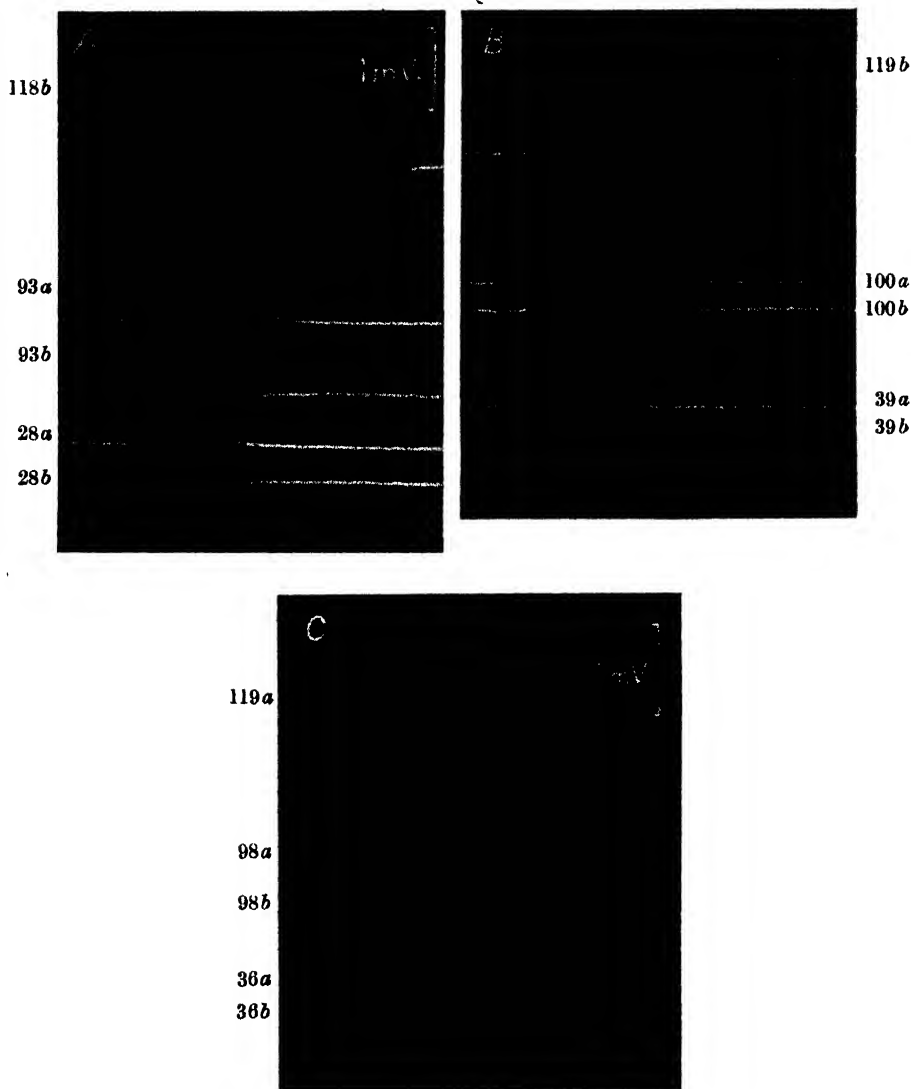
Fig. 2. Potential changes in frog's nerve due to brief shocks, recorded with set-up of Fig. 1. Negativity at lead *C* gives upward deflexion. *A* and *B*, sciatic nerve at 24° C. Two different positions of lead *C* (cf. Fig. 1). Several sweeps are superimposed. Opposite polarities of shocks are indicated as (*a*) and (*b*). Strength of shocks in relative units (the strength at which least diphasic spike was seen is taken as 100). *A*, successively from above: 118 (*b*), 93 (*a*), 93 (*b*), 28 (*a*), 28 (*b*). *B*, 119 (*b*), 100 (*a*) (subthreshold), 100 (*b*) (threshold, a small diphasic spike appears occasionally), 39 (*a*), 39 (*b*). *C*, another sciatic nerve at 23·5° C. Single sweep records, from above: 119 (*a*), 98 (*a*), 98 (*b*), 36 (*a*), 36 (*b*). Time base, 5 kcyc./sec.; voltage scale, 1 mV.

nerve. The initial artifact lasts about 0·1 msec., sometimes less, depending upon the effectiveness of the balancing arrangement. After that, the records can be measured accurately and scrutinized for any 'non-linear' negative potential change. The crucial test is whether, on reversing the shock, the residual potential recorded at C reverses symmetrically, or whether there is an asymmetrical change which has the characteristics of a local response.

The results are illustrated in Figs. 2 and 4. It is seen that with weak stimuli, of 0·3 threshold and less, there is an almost perfect symmetry of the local
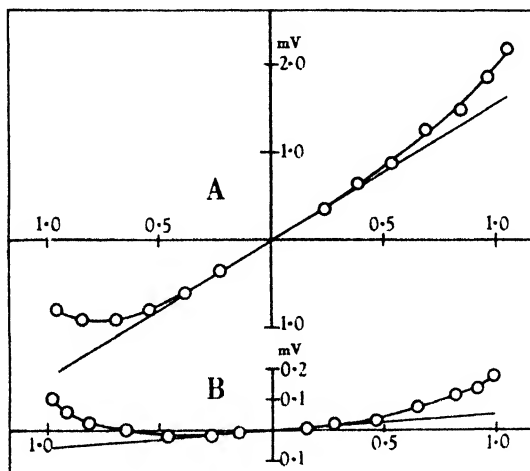
Fig. 3. Local potential changes in frog's sciatic nerve. A and B, two typical experiments, with different degrees of 'shock balance'. Ordinates: local potential change at a given brief interval after the shock (0·1–0·15 msec.), negativity plotted upwards. Abscissae: shock intensity, in relative units, threshold of least diphasic spike being taken as unity.

potential changes, while with stronger, but still subliminal shocks, a marked asymmetry appears which is due to a progressively increasing local depolarization.

If the potential is measured at a given time after the beginning of the shock and plotted against shock intensity, a characteristic curve is obtained which is approximately linear over a small range of weak intensities and, from about 0·3 threshold bends *up* towards increased negative potential independently of the direction of the current. This suggests that, from about 0·3 threshold onwards, a non-linearly increasing depolarization is added to the unbalanced remainder of shock and electrotonic potential. This was verified by slightly altering the position of lead C, thus causing a variation in the size and shape of shock and electrotonic potential, without affecting that of the superimposed non-linear depolarization (cf. Fig. 6). Hence, the slope of the linear portion in Figs. 3 and 4, A is quite arbitrary and depends merely upon the adjustment of the balancing electrode: with perfect balance, the initial slope of the curve

would be zero. If we extrapolate this linear portion, we may use it as a base-line from which to measure the size of the non-linear effect. In seventeen experiments on the frog's sciatic nerve, at about 20° C., the mean size of the local depolarization at threshold strength was 0·37 mV., varying between 0·1 and 0·75 mV.
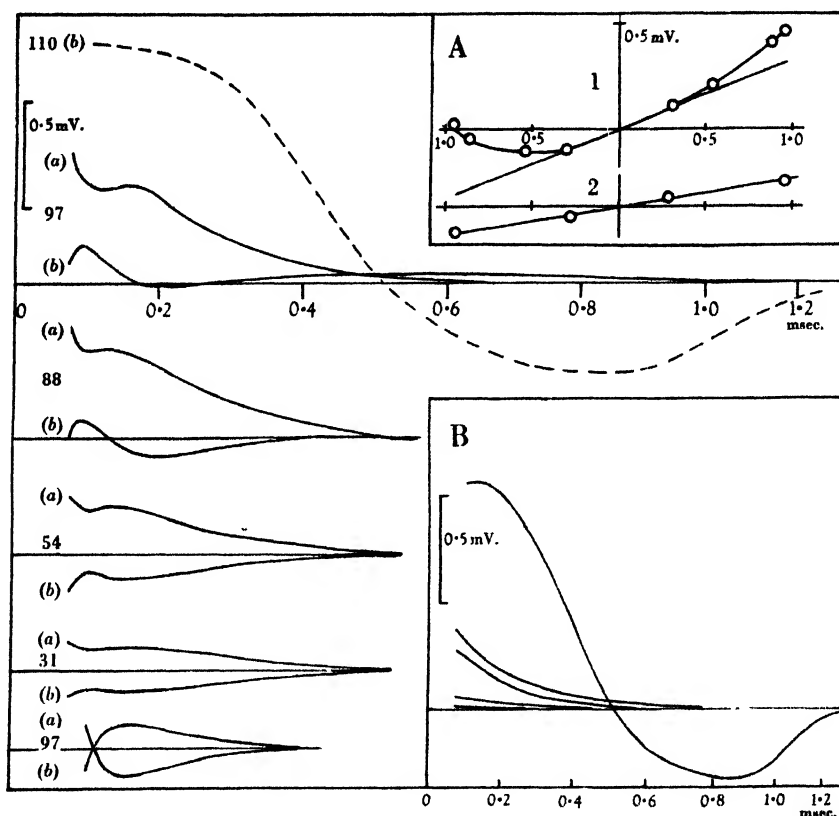
Fig. 4. Sciatic nerve at 23·5° C. Potential changes with equal and opposite shocks. Strength and polarity of shocks indicated as in Fig. 2. From above: 110 (*b*) (broken line); 97, 88, 54 and 31, (*a*) and (*b*) each. Lowest record: strength 97 repeated after crushing. Inset *A*, local potential changes plotted as in Fig. 3, at 0·16 msec. after beginning of shock. A 1: normal nerve; A 2: after crushing. Inset *B*, mean potentials, with equal and opposite shocks. Successively from above: 110; 97, 88, 54, 31. After crushing, the mean potentials become indistinguishable from the baseline.

It is clear that this local negative potential change is of the same origin as that previously recorded with a slow, integrating galvanometer (Katz, 1937, Fig. 16). In both cases, the negativity became measurable above about 0·3 threshold and increased progressively with stimulus strength. The galvano-meter records, however, did not permit size or time course to be determined;

the integrated negative potential, with subthreshold alternating current at 5000 cyc./sec., was equivalent to a permanent potential change of about 50–100 μV.

To find the duration of the local depolarization, the mean of two potential changes, with shocks of equal strength and opposite sign, must be determined, as shown in Figs. 4 and 5. In most experiments, the initial part of the curve was obscured by shock artifact, and only its falling phase could be determined. The decline is approximately exponential with a half-time of about 115 μsec. (This low value applies to a small interelectrode distance, and a temperature of 23° C.) In the case of Fig. 5, a local depolarization of 0·3 mV. is obtained which rises to a peak in 65 μsec. and declines with a half-time of 105 μsec. These values are slightly too large because of a small lag introduced by the amplifier (see 'Methods'). The time course may be compared with that of the 'local response' predicted on the basis of excitability measurements (Katz, 1937, Fig. 11). It was indicated there, that for small interelectrode distance (1–2 mm.) and at room temperature, the local response with a 95 % threshold stimulus rises to a peak in 80 μsec. and falls with a half-time of 115 μsec. With a stimulus of 85 % threshold, the rising phase would be shortened to about 60 μsec. (cf. Katz, 1937, Fig. 4, *A*).

It was usually found that with increased stimuli, the local depolarization increased in size as well as duration (Figs. 4 and 6), which again agrees with the earlier analysis of excitability measurements (Katz, 1937).

Extensive checks were made to reveal sources of error which might vitiate these results. The non-linear depolarization is abolished by deep alcohol narcosis, or by crushing the nerve, and a strictly linear artifact only remains (Fig. 4). Other routine tests were made (varying the amplification; reversing amplifier input or output leads to check against non-linearity in the recording system; varying the position of the earth point; altering the position of the recording leads to obtain different degrees of unbalance; using non-polarizable electrodes), but they failed to reveal any significant fault.

The question arose whether the depolarization was, in fact, a subliminal and non-conducted change, or possibly due to fibres which were blocked or cut between the recording leads, or for some other reason did not give a diphasic action potential.

In the first place, however, the 'minimum threshold' indicated by the sudden appearance of a diphasic spike, is usually well defined, and can be determined within about 5 % of stimulus strength, even on the whole sciatic nerve and with moderately high amplification (see Fig. 2). The critical threshold value can then be verified by using higher gain. At amplifications of about 10^6, the 'threshold play' of single, or a few synchronous, spikes can be seen in many sciatic nerves quite clearly and leaves no doubt as to the critical threshold of the most excitable fibre, or group of fibres. As the 'non-linear

effect' becomes measurable with stimuli of little more than one-third of that strength, conducted impulses can hardly be involved.
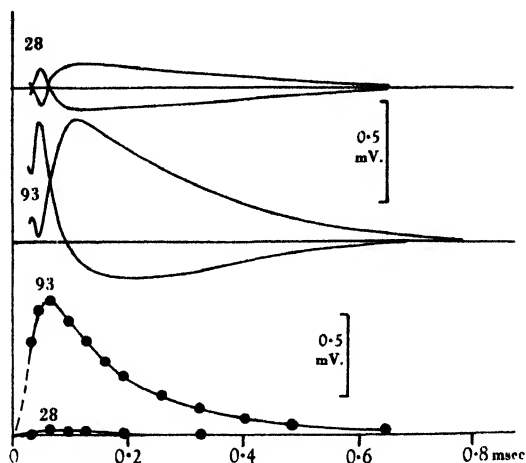
Fig. 5. Sciatic nerve, 24° C. From above: local potential changes with shock intensities 28 and 93, and mean potentials.
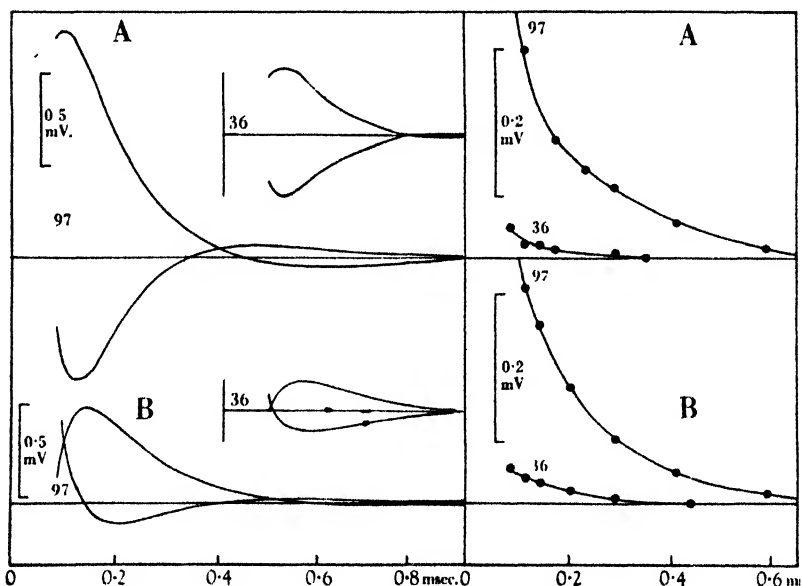
Fig. 6. Effect of varying the balancing position of lead *C* (cf. Fig. 1). Left part of figure shows recorded potential changes with strength 97 and 36. Different balancing positions in *A* and *B*. Right part shows the respective mean potentials, which are not appreciably affected by altering the balancing point.

Moreover, the non-linear negativity shows a typical spatial decrement: if the distance between lead *C* and the stimulating electrodes is increased to

about 2 mm., the local depolarization at C is reduced to about two-thirds, and if the distance is made greater than 6 mm., it is abolished.

One may, therefore, conclude that in the frog's sciatic nerve, subliminal stimuli produce a potential change which has the characteristics of a local response and which, in its electric sign, its non-linear growth and relation to threshold strength, in its time course and spatial spread, corresponds with the local responses described in earlier papers.

Several experiments were made on the peroneal and phalangeal branches of the sciatic nerve, but these gave much less clear-cut results. In two experiments on peroneal nerves, a local response of 0·3 and 0·1 mV. was obtained. In two out of six experiments on phalangeal nerves, a non-linear depolarization was seen with certainty (0·1 and 0·05 mV. respectively); in the other cases, local responses were either absent or so small as to be of doubtful significance.

This discrepancy between different nerves may be due to the fact that the aggregate spike potential of the phalangeal nerve is much smaller than that of the sciatic (e.g. at twice threshold strength, one obtains 3–4 and 15–20 mV. respectively) and that the phalangeal nerve contains relatively few large fibres (Blair & Erlanger, 1933) whose thresholds, moreover, are widely separated. It is true that the minimium transmitted response in the thin phalangeal nerve is fairly large and discrete, giving an all-or-none spike of about 0·25 mV., but a 10 or 20% stronger shock may be needed to bring in more fibres. In the sciatic nerve, although the threshold response is very small, a large number of axons are brought in at a 10 or 20% greater intensity, giving a much larger spike, and all these fibres are responsible for a portion of the subliminal 'local response'. As the applicable stimulus intensity is limited by the fibres of lowest threshold, the chances of seeing a local response in a compound nerve are small unless it contains many, fairly uniform, large fibres, whose individual thresholds are not too far apart. The local response due to one or two large fibres only, giving a spike of 0·25 mV., may be no more than 20μV., and this is not large enough to be recorded with certainty. That this explanation is probably correct was suggested by an experiment on a sciatic nerve which contained a few unusually excitable fibres, their threshold being separated by a large step from the rest of the nerve. In this nerve, only a very small local response could be seen, as the applicable stimulus strength was limited by the threshold of the hyperexcitable fibres and, therefore, was very weak relative to the majority of the fibres. The matter has been discussed at some length, as it has probably some bearing on Blair's (1938) consistent failure to observe a local response in the frog's phalangeal nerve.

The result of this investigation is in complete agreement with the findings previously described (Katz, 1937, 1939 a) and shows that the non-linear increment of excitability at the cathode is accompanied by a brief depolarization of the same temporal and spatial characteristics. The size of this local response, relative to the full-grown spike, cannot be determined in multi-fibre preparations, but it is probably rather less than 10 % of the individual fibre spike.

The present observations are in contrast with statements by Blair (1938) and Tasaki (1940) disclaiming the existence of any non-linear effect of sub-threshold stimuli on medullated axons. Blair worked exclusively on the frog's phalangeal nerve where the chances of observing a local response are small. He used a common recording and stimulating lead of about 2 mm. effective width (cf. Blair & Erlanger, 1936), which would reduce the local potential changes owing to electrotonic decrement.

Even so, Blair's consistent failure to observe any non-linear effect might have had something to do with his particular method of testing for it (Blair, 1938, Fig. 2). By *halving* the intensity of a near-threshold shock and *doubling* the amplifier gain, he obtained two records, which matched well during their later part, but differed during the initial shock artifact, which was consistently larger with the weaker shock and higher gain. Blair dismissed this discrepancy as insignificant and ascribed it to an artifact. This may be justified, but as an alternative possibility, the discrepancy may have been due to a small calibration error causing the shock to be a little more than one-half, or the amplifier gain slightly more than double, which would significantly affect his argument.

Tasaki (1940) emphatically denied the existence of a local response, though it is not clear on what particular piece of evidence that statement was based. In a more recent paper, Tasaki & Takeuchi (1942) admit the possibility of a partial excitation of the nerve membrane, but claim that according to 'theoretical considerations' the accompanying potential would be too small to be seen. No calculations are given to support this statement which seems to depend upon faulty speculations. Working on an isolated frog's nerve fibre, Tasaki & Takeuchi (1942, pp. 765–8) actually record potential changes which have the characteristics of a local response (non-linear increase up to threshold; duration about 0·5 msec.; size nearly one-sixth of a 'uni-nodal' spike which, at threshold, takes off from it; abolished by urethane). Tasaki & Takeuchi conclude, however, that this cannot be a local response, because according to their theory, the latter should be immeasurably small. As an alternative, they suggest that the subthreshold potential change is due to 'action currents of the connective tissue' or perhaps of some invisible sympathetic fibres, but one may be permitted to wonder whether the authors themselves take this strange argument very seriously. Invisibly small residual nerve fibres would neither

have the required low threshold, nor give a fair-size action potential, compared with the isolated motor axon; and connective tissue which gives action potentials indistinguishable from a local response of the nerve, still remains to be discovered. If not their interpretation, their experimental result itself, obtained on a skilfully dissected single motor axon, certainly falls into line with all the other positive evidence of a non-conducted response and is, in fact, an excellent confirmation.

Tasaki & Takeuchi (1942, Fig. 2) measured a 20 % lowering of threshold at an adjacent node of Ranvier, accompanying the local potential change. They regard this as unduly small and believe that there is no connexion between potential and excitability change. Considering, however, that the local action potential was less than one-sixth in amplitude, and less than one-eighteenth in potential-time area, than the spike, it is difficult to follow their argument.

An interesting feature of the local response is its relation to stimulus intensity (Fig. 3). There is no definite point at which the local response starts, but merely a small initial curvature which becomes noticeable at about 0·3 threshold. Apparently we are dealing, not with a membrane response of critical threshold, but with some continuous non-linear function of the nerve membrane which eventually, at propagation threshold. leads to a self-regenerating or 'chain' reaction (cf. Appendix).

Local depolarization is not the only non-linear effect produced by subthreshold stimuli. It has been shown (Cole & Baker, 1941; Katz, 1942) that the impedance of nerve and muscle is a continuous, non-linear function of the applied current, and that the membrane conductance increases at the cathode as the resting potential is diminished and, vice versa, decreases at the anode. It is natural to suppose that the two non-linear processes, viz. changes of membrane conductance, or ion permeability, and of subthreshold potential, are somehow related. A simple reaction by which they might be linked, and which might lead to a progressive chain-reaction, has been outlined elsewhere (Katz, 1942).

It must be pointed out that the presence of a non-ohmic membrane resistance which is reduced at the cathode does not, *in itself*, lead to an excess depolarization. On the contrary, a mere reduction of resistance at the cathode would cause the catelectrotonic potential to be *smaller* than the anelectrotonic. One would have to assume some secondary change (cf. Katz, 1942, p. 180) to find a causal connexion between the two phenomena.

SUMMARY

1. Blair (1938) and Tasaki (1940) have insisted that the nerve impulse in normal medullated axons is generated in an all-or-none fashion, that the effects of subliminal shocks are strictly proportional to their intensity, and that a local response to a subthreshold stimulus does not exist.

2. The matter has been re-examined by recording the subthreshold potential changes in the frog's sciatic nerve, resulting from brief single shocks. Using a small distance between the stimulating electrodes and a bridge-like arrangement of the recording leads, it is possible to reduce shock artifacts and electrotonic potentials sufficiently to search for a non-conducted response.

3. With weak shocks, up to about one-third of threshold, the local potential change (i.e. unbalanced remainder of shock and polarization potential) increases linearly with shock intensity. Above that strength, a small local depolarization is observed which increases more than linearly as the stimulus intensity is raised. At threshold, the amplitude of this local negative potential is about 0·35 mV. (mean of seventeen experiments) in the sciatic nerve. This potential is abolished by killing or deeply narcotizing the nerve.

4. The time course of this depolarization is brief: at 24° C. and with an interelectrode distance of 1·5 mm. it rises to a peak in about 65 μsec. and falls approximately exponentially, with a half-time of about 110 μsec. This compares well with the predicted time course of the 'local response' previously obtained by analysis of excitability curves.

5. The results confirm previous findings indicating the existence of a local response to subliminal stimuli in medullated nerve. The experimental basis of contrary statements in the literature is discussed.

APPENDIX

To illustrate what is meant by 'partial excitation' and the 'chain reaction' mentioned above, the following model may be considered (Fig. 7). The membrane element r ('ion permeability') is assumed to vary as a function of the potential p across the membrane and to have the non-linear characteristic shown in Fig. 8, A. All other components have fixed values. The resting membrane has a resistance r_0 and a potential difference p_0. It is clear from Fig. 7 that any change of r will be followed automatically by a change in p, while—according to Fig. 8, A—any change of p will also cause a change of r. At a certain point of the curve, depending upon its attaining a critical slope, the process becomes 'explosive'.

Fig. 7. Electric model of a membrane element.

Let us consider the case of a very brief shock, by which the membrane charge is instantly altered and then left to itself, without any external current flowing. The rate at which the membrane potential changes, after the shock, is then given by

$$-C\frac{dp}{dt}=\frac{p}{r}+\frac{p-E}{R},\qquad (1)$$

where r is a function of p. To simplify calculations, we assume R to be large compared with r. We then obtain $p_0 = Er_0/R$, and from equation (1)

$$C\frac{dp}{dt} = \frac{p_0}{r_0} - \frac{p}{r}. \tag{2}$$

In Fig. 8, B, dp/dt is plotted as a function of p. It is seen that as the membrane potential p is reduced below the resting value p_0, it tends, at first, to
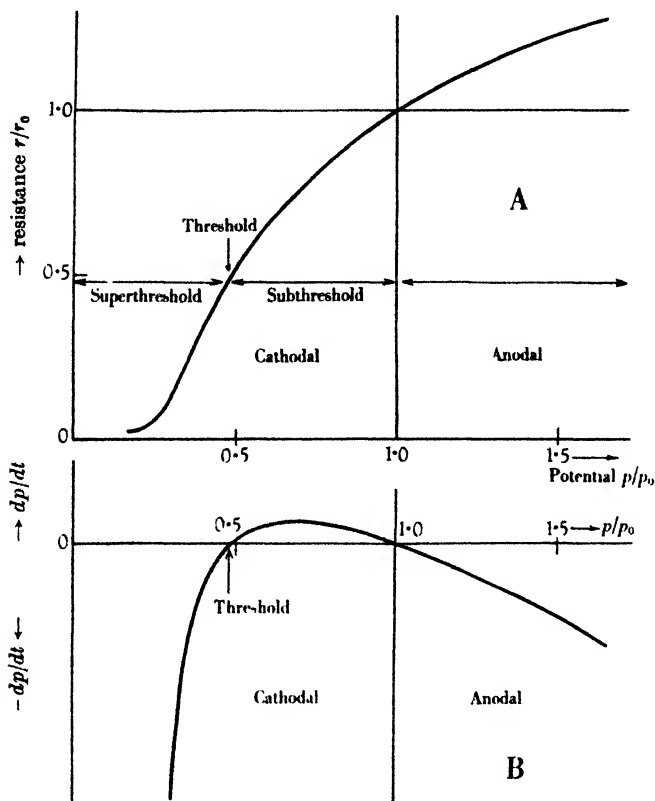
Fig. 8. A, assumed relation between r and p; B, rate of potential change dp/dt plotted against p.

return to the resting level. Beyond a certain point, this return becomes slower; at a critical point ('threshold'), the depolarization maintains itself and, a little beyond it, becomes progressive. If the membrane potential has been raised above the resting level (at the anode), it always tends to return to it.

Equation (2) can be integrated, e.g. by using an empirical formula expressing r as a function of p (Fig. 8, A) and operating in small successive steps. One then obtains a family of curves showing the potential changes following brief shocks of various intensities (Fig. 9). It is clear that these curves are very similar to those described by Hodgkin (1938) and Katz (1937).

Needless to say, the present scheme suffers from the usual limitations of an *ad hoc* model, in that it illustrates only one particular aspect of electric excitation. It takes no account of recovery or of steady state phenomena, nor of the fact that the action potential is greater than the resting potential (Hodgkin & Huxley, 1945).
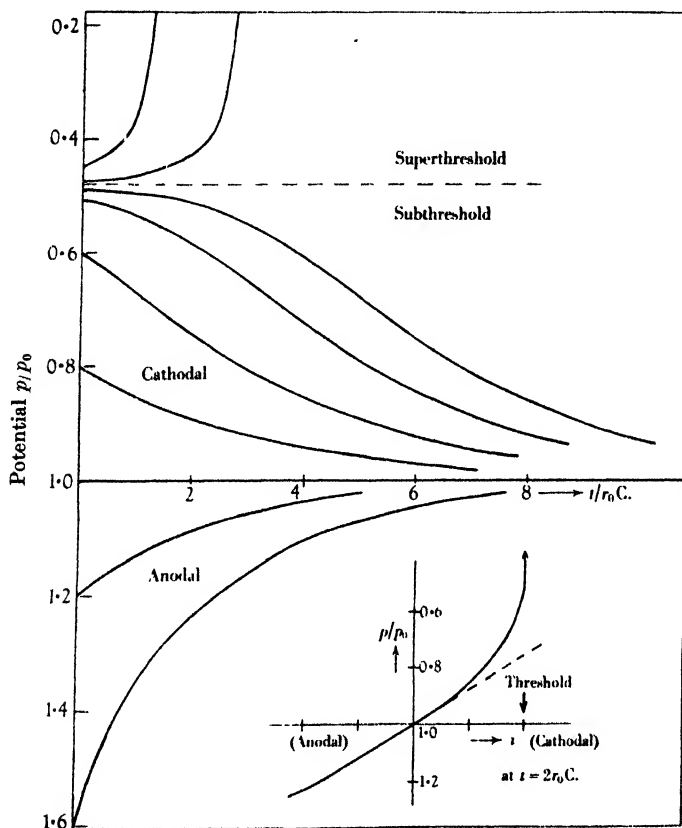
Fig. 9. Time course of membrane potential following brief shocks of various intensities. Inset, potential change, at a given time interval ($t = 2r_0 C$) plotted against relative shock intensity.

There is another objection. Impedance measurements with alternating current (Cole & Baker, 1941; Katz, 1942) have shown that the membrane resistance decreases at the cathode, when an applied stimulus approaches threshold. But Fig. 9 (inset) shows a relation between voltage and current which curves upward, and this would seem to require that the membrane resistance, as a whole, should increase at the cathode. As Fig. 9 agrees with the data obtained by Hodgkin (1938) and others, there seems to be a curious conflict between the two sets of observations. It should, however, be borne in mind: (*a*) that the relation between an applied current and a transient potential

change is not the same as an impedance measurement, made during the potential change, with alternating current of relatively high frequency, and (*b*) that, in the case of Fig. 9 (inset), *i* refers to a brief initial pulse and *p* to a potential change measured after a certain interval, so that little light is thrown on the value of the membrane resistance. Nevertheless, there remains an apparent discrepancy which has not been satisfactorily resolved.

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ACTION OF INTRATHECALLY INJECTED ESERINE
ON THE SPINAL CORD OF THE CAT

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General agreement on the central action of eserine has not yet been reached, as the results of different investigators have not been concordant. Table 1, which summarizes the literature, suggests that the animal species, the anaesthetic, the type of preparation, the route of administration, the dose of the drug and the level of the neuraxis affected by it, may modify, or even reverse, the action of eserine on the central nervous system.

TABLE 1. Summary of literature on effects of eserine on the central nervous system

Author	Year	Species	Preparation	Dose and Route	Reaction studied	Results + potentiation − depression
Schweitzer & Wright	1937*b*	Cat	Chloralose	0·1–0·5 mg. intravenous	Knee jerk	+ (occasionally −
Bonnet & Bremer	1937	Toad, frog	Spinal	50–200 μg. intravenous	Homolateral reflex twitch of semitendinosus muscle and after-discharges	After-discharge +
Bremer, Bonnet & Moldaver	1942					
Merlis & Lawson	1939	Dog	Na barbital or chloralose anaesthesia	0·01–2 mg. intravenous or intrathecal	Knee jerk Flexor reflex	+ or − +
Chute, Feldberg & Smyth	1940	Cat	Perfused head, almost completely isolated	1 : 300,000– 1 : 400,000 in perfusion fluid	General reflex excitability Blinking reflex	+ +
McKail, Obrador & Wilson	1941	Cat	Dial or urethane anaesthesia	0·1–0·5 mg. intracarotid	Flexor reflex	+
Bülbring & Burn	1941	Dog	Chloralose anaesthesia. Isolated, perfused spinal cord	0·2–5 mg. intra-arterial	Knee jerk Flexor reflex	− +
Kremer	1942	Man	Spinal block	0·25–1 mg. intrathecal	Reflex activity Muscle tone Deep reflexes	+ (initial −) + (initial −) + (initial −)
Calma & Wright	1944	Cat	Decerebrate	1–4 mg. intravenous	Muscle tone of extensor muscles	+

We have investigated the action of eserine injected intrathecally in cats. This method of administration was chosen to eliminate as far as possible the action of the drug on systems other than the central nervous system, and to obtain a high concentration of the drug on the structures under investigation, i.e. the whole of the neuraxis in the anaesthetized cat, the brain stem and spinal cord in the decerebrate cat, and the caudal part of the cord in the spinal animal.

We have found that, under these conditions, eserine regularly increases the reflex (excitatory) responses of the extensor motoneurones of the spinal cord, but that its action is more variable on flexor reflexes. Some evidence of a central inhibitory action of eserine was also obtained.

METHODS

Experiments were performed on the cat under chloralose anaesthesia and on the decerebrate animal.

Chloralosed cat

The animals were anaesthetized with chloralose (0·08 g./kg. intravenously). The nerves to the muscles antagonistic to those reflexly stimulated were severed: in the case of the knee jerk and crossed-extensor reflex, the sciatic nerve high up in the thigh and, in many cases, the nerve to the sartorius muscle and the saphenous nerve were cut; in the case of the flexor reflex, the femoral nerve, the nerves to the hamstring muscles and often the tendon of the ileopsoas were divided. The knee jerk was elicited at regular intervals (every 9 or 10 sec.) by means of an electromagnetically operated tapper (Schweitzer & Wright, 1937a); the flexor and the crossed-extensor reflexes by single break-shocks applied at 9 or 10 sec. intervals to the central end of the cut tibial nerve through fluid electrodes of the Collison type. When both the knee jerk and the flexor reflex were elicited, the knee jerk was elicited first, and the stimulus for the flexor reflex was applied after an interval of 0·6–1 sec. The knee jerk was recorded by connecting the ankle to a torsion lever myograph by means of a thread running over a pulley, the crossed-extensor reflex was recorded in the same way or else by connecting the patellar tendon directly to the myograph. The flexor reflex was recorded directly from the tendon of the tibialis anticus. Drills were passed into the upper and lower ends of the femur and tibia to fix the preparation.

Eserine was injected into the theca through a blunt metal cannula inserted into the subarachnoid space at the level of the 7th–8th post-thoracic vertebra, i.e. 2–2·5 cm. below the lumbar enlargement. The spinal cord was exposed, care being taken not to damage the dura and the nerve roots. The dura was pierced with a sharp, large French needle. This was withdrawn and the blunt cannula inserted, tied in position with a thread passed externally round the dura and finally fixed with ligatures passed through the muscles of the back.

Some animals were secondarily made spinal. Simple transection of the spinal cord at the required level was not employed, as that would have opened up the subarachnoid space and allowed the rapid escape of intrathecally injected eserine; instead, the spinal cord was tightly compressed to produce a physiological block of its long tracts. The spinal cord at the level of the proposed block was exposed, a thread was passed round it enveloping the meninges, and very firmly tied around the dura and spinal cord. We satisfied ourselves by the following criteria that the block produced in this way was complete: the cord inside the ligature appears crushed; in the decerebrate animal the hindlimbs become flaccid, while the forelimbs remain rigid and extended; the effects of injected eserine are limited entirely to the lower limbs. It may, therefore, be assumed that this type of block prevents impulses from passing up or down the spinal cord at the level of the ligature, and limits the local action of intrathecally injected eserine to the spinal cord caudal to the level of the ligature.

The eserine sulphate or salicylate was dissolved in acid (pH 4–4·5) or in neutral saline. The volume of fluid injected varied, the range being 0·1–2 c.c. Atropine was not used. Control injections of similar volumes of acid and neutral saline were always carried out before and after administration of eserine, until the control effects were well established.

Decerebrate cat

Under ether-chloroform anaesthesia, the limbs were suitably prepared and, when desired, spinal block was produced as in the chloralosed preparation. The animal was then decerebrated at the level of the anterior colliculi, or occasionally more cranially. The cannula was inserted into the theca and the electrodes applied to the nerves to be stimulated. The preparation was rested for ½–1 hr., so that the anaesthetic might be blown off and a steady state reached.

Control experiments were carried out to see whether eserine, in the dose and by the route employed, modified muscular contractions by a peripheral action, i.e. whether the amount of eserine which might be absorbed was sufficient to influence the peripheral muscular responses. Two procedures were used: (a) simultaneous records of the reflexes and of the contractions of the gastrocnemius muscle stimulated through its motor nerve (Fig. 1 A–G, upper records); (b) ischaemic preparations in which access of the drug to the muscles of the limbs was prevented by depriving the limbs of their blood supply, according to the technique devised by Schweitzer & Wright (1937c). We obtained no evidence that any significant peripheral actions were produced.

<div style="text-align:center">RESULTS</div>

Control experiments were carried out by intrathecally injecting either acid or neutral saline (0·2–2 c.c.), slowly or quickly, at room temperature or at 37° C. The effects thus produced on the activity of the spinal cord were irregular and transient; if less than 0·5 c.c. of fluid was injected slowly no significant changes occurred (Fig. 1 A).

Chloralosed cat

In such animals, especially when the spinal cord has been tied (and thus transected) at Th. 9, intrathecal injections of physiological saline may produce an immediate, small, and brief contraction of the innervated muscles of the hindlimbs; a small temporary increase of tone and of the reflex contraction under examination often also occurs. After about 30 sec., however, the reflexes return to their initial level or show a secondary depression. In some cases, after the intrathecal injection of large volumes of saline, e.g. 1·0–2·0 c.c. (and especially when the injection was made rapidly), we observed an immediate and very marked rise of blood pressure, which was sometimes 100 mm. Hg in extent. This increase in blood pressure may be accompanied by an increase in the heart rate and development of extra-systoles. Similar vascular changes occurred with similar frequency when eserine was injected. The characteristic effects of eserine on the reflexes were independent of the occurrence or absence of these vascular changes. The responses obtained are independent of the reaction of the fluid employed, and are therefore mechanically produced. These effects are considered fully in another paper (Calma & Wright, 1947).

Decerebrate cat

In the decerebrate cat with spinal cord intact, the mechanical effects of intrathecal injection of large volumes of saline are more regularly observed (Fig. 2). In decerebrate preparations made spinal, intrathecal saline injections of the order of 1·0-2·0 c.c., given rapidly, cause larger changes in tone and reflexes, as might be expected if rise of intrathecal pressure were the causal factor. Thus, in one experiment, the tone of the tibialis anticus was increased, the flexor reflex was almost doubled in extent, and the knee jerk after a short delay was temporarily abolished; after a longer delay the flexor reflex was depressed for some time and the small crossed-extensor reflex disappeared. When the fluid is injected slowly and its volume does not exceed 0·5 c.c., the mechanical effects are much reduced or are absent.

With saline injections, however, the preparation, after 1 min. or so, settles down to a steady state, approximating to that present before the injection, or showing some lasting depression. In view of these results, changes occurring during the first minute after the intrathecal injection of eserine have not been regarded as significant. Following the injection of eserine, as will be explained below, after a variable further latent period, characteristic changes make their appearance, and they can be distinguished from the immediate mechanical effects by the intervening 'normal' period, and also because changes of the type noted do not occur after saline injections.

Observations on the knee jerk

Preparations with spinal cord intact

Effects of eserine in the chloralosed cat. Eserine salicylate or sulphate injected intrathecally in doses of 20-100 μg. has no effect on the size or shape of the knee jerk in most preparations and does not produce 'spontaneous' discharges. In a few instances, repeated injections of small doses of eserine (20 + 30 + 100 μg.) at intervals of 3-5 min., prolonged the relaxation phase of the knee jerk without any change in the peak tension of the reflex. Doses of eserine of 0·2 mg. or more produced regularly, after the initial mechanical effects (if any) had passed away, the following effects on the knee jerk: increased tension of the reflex contraction, alterations in the shape and duration of the relaxation curve, increase of the initial level of tension in the quadriceps muscle, irradiation of the reflex response to neighbouring muscles. The characteristic effects set in after a latent period of about 2-2½ min. from the onset of the injection, which was usually rapidly completed, i.e. in 2-5 sec. They develop gradually and reach their maximum intensity after 3-5 min. The total duration of the changes varies widely in different preparations: the maximum duration observed was 20 min., the shortest 5 min. The effects wear off gradually.

The maximal increase in the tension of the knee jerk was as much as fourfold in some preparations; in others the increase was only 30 %. There seems to be
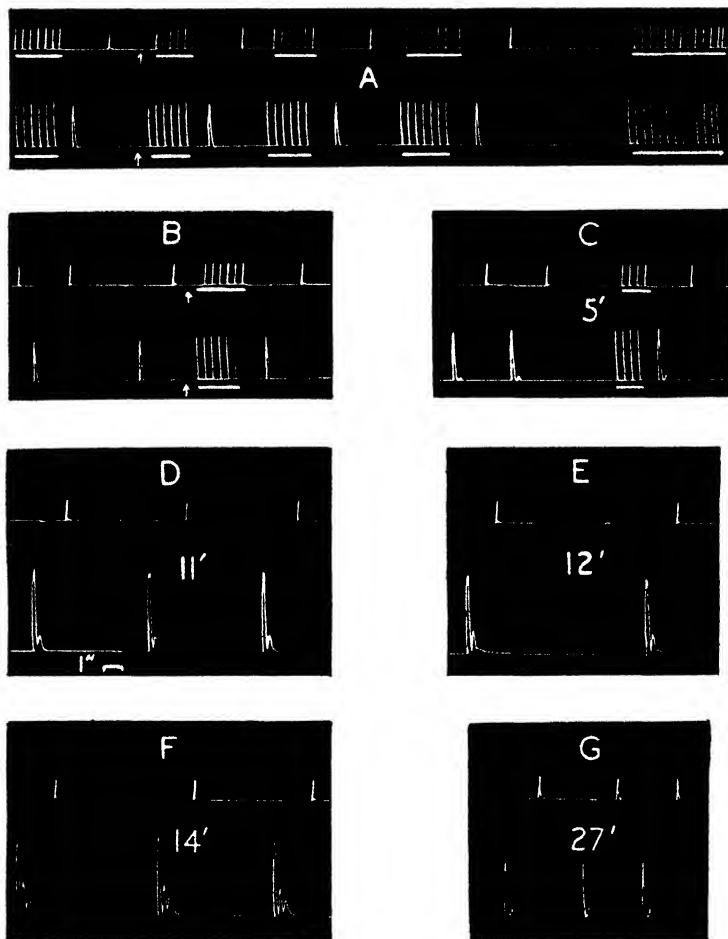
Fig. 1. Cat. 2·4 kg. Chloralose. In each section of the tracing, upper record is contraction of left gastrocnemius stimulated through its motor nerve; lower record is right knee jerk elicited by taps on right patellar tendon. The parts of the tracing taken on a slow drum are underlined, and the intervals between the individual contractions represent 10 sec. Otherwise the fast drum was employed, the time scale for which is given in D. A. Control; at arrow inject 0·5 c.c. of saline intrathecally. B. At arrow inject 0·3 mg. of eserine sulphate in 0·5 c.c. saline intrathecally. C, D, E, F, G were recorded 5, 11, 12, 14 and 27 min. respectively after the injection of eserine.

no relationship between the increase in tension and the increase of the after-effects as depicted by the character of the relaxation curve; in some instances the predominant effect was on the peak tension, with only a modest increase in the after-effects, while in other cases the condition was reversed. Fig. 1 (lower

records) illustrates some of the points to which reference has been made. After 5 min. (Fig. 1 C) the amplitude of the knee jerk has increased slightly; relaxation is followed by one secondary contraction. After 11 min. (Fig. 1 D), the tension of the contraction is doubled, and its duration is prolonged; relaxation is initially not complete, but is interrupted by a secondary contraction which is larger than in Fig. 1 C. After 12 min (Fig. 1 E) this secondary contraction lasts still longer and the beginning of a further after-contraction is visible. In Fig. 1 F (14 min.) two very well-developed after-contractions are seen. In Fig. 1 G (27 min.) the effects have to a large extent passed away.

The changes observed are not due to a peripheral action of absorbed eserine on the muscles. The gastrocnemius muscle stimulated through its motor nerve shows no changes whatever (upper records Fig. 1 A–G). Results similar to those described have been obtained in 'ischaemic' preparations in which the blood supply to the hindlimbs had been temporarily cut off. As eserine in these concentrations has no effect on peripheral nerves, the results can be confidently attributed to an action of eserine on elements in the spinal cord. The increase in the tension of the knee jerk must be due either to an increase in the frequency of the discharge of the affected motoneurones, or to recruitment of additional motoneurones, or to both changes occurring together. In either case they indicate increased excitability of the spinal cord, e.g., additional delay paths being opened up, and/or regions of subliminal stimulation becoming liminally excited.

The after-effects (delayed relaxation, secondary contractions) must be ascribed to after-discharge, in the sense that they occur after the afferent impulses set up by the patellar tap and travelling along the direct route to the motoneurones have died down. We have not determined how much of the after-effects is due to the 'back-lash' from the contracting quadriceps itself and how much is due to a greater true central after-discharge, independent of such fresh afferent impulses. Whatever the intimate nature of the mechanism, the duration of the discharge of the motoneurones is greatly increased. Bonnet & Bremer (1937) showed that eserine in the frog and toad increased the after-discharge of the semitendinosus muscle after ipsilateral stimulation, even when the initial contraction was unaffected or depressed, and after section of posterior nerve roots containing the proprioceptive afferents from the muscle. Irradiation of the reflex to adjacent muscles was not graphically recorded, but was frequently noted by us in the muscles of the anterior abdominal wall of one or both sides. General convulsions (as described by Schweitzer & Wright (1937 b) following intravenous injection of eserine) were not observed.

Effects of eserine in the decerebrate cat. In a previous paper (Calma & Wright, 1944) it was shown that intravenously injected eserine in doses of 1–4 mg. markedly increases the tone of the extensor muscles in the decerebrate cat. In this series of experiments, in which eserine was injected intrathecally,

similar results were obtained (Fig. 2). The latent period is similar to that of the chloralosed preparation, i.e. about 2 min. In Fig. 2, after the initial transient changes of mechanical origin, each successive individual knee jerk is followed by a well-marked shortening reaction. The resting tone of the quadriceps muscle between stimuli thus rises in a step-like manner, while the knee jerks become progressively smaller. The new base line between any two consecutive knee jerks runs absolutely level. After 130 sec. the shortening reaction develops at the peak of the knee jerk, so that the maximal tension obtained during the jerk is maintained until the next reflex is produced. Ultimately a
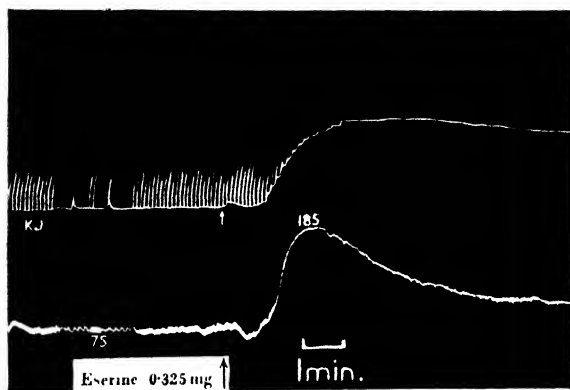
Fig. 2. Cat. 4·8 kg. Decerebrate. Records from above downwards: knee jerk, arterial blood pressure. At arrow inject 0·325 mg. of eserine sulphate intrathecally. The injection is followed by a small initial transient increase of the knee jerk and quadriceps tone (which is an effect of the volume of fluid injected) and is followed by the specific eserine effect.

plateau develops which shows only slight irregularities and is maintained at a fairly steady level for 14 min. and then begins to decline slowly. Intermission of one or several stimuli to the patellar tendon did not significantly alter the character of the curve at this stage.

The changes described seem to indicate progressive irradiation of the reflex maintaining quadriceps tone, together with increasing occlusion of the knee jerk. Ultimately, reflex tone alone activates a larger pool of extensor moto-neurones than came originally under excitation from the combined effects of reflex muscle tone and knee jerk.

Another type of reaction was observed in some preparations. Before the injection of eserine, the base line on which the knee jerk was inscribed, runs horizontally, without any change of level; 2-3 min. after administration of eserine the base line shows irregular and unpredictable waves on which smaller knee jerks are inscribed. In other words the muscular tone after eserine shows temporary increases which were absent before eserine, accompanied by some degree of occlusion of the knee jerk. In all the decerebrate preparations with

spinal cord intact, the predominant features of the response, namely the increase in tone and the associated disappearance of the knee jerk, do not allow an analysis either of the shape of the knee jerk or of the after-discharges.

Preparations with spinal block

Effects of eserine in the chloralosed cat. Eserine, injected intrathecally into preparations with spinal block at Th. 9, has the same action as in preparations with the spinal cord intact. In order to localize more exactly the site of action of eserine, we performed a few experiments in cats with spinal block at Th. 13, L1 and L2. In four out of five experiments eserine produced no effect on the knee jerk (or on the blood pressure). The 'immediate' reflex centre must have been intact because the knee jerk could still be elicited. Spinal shock, which might have been produced by the manipulations and by the actual ligature used to produce the block, must be considered; it is well known, however, that spinal shock does not occur readily in cats and is, in any event, a quite temporary condition. In two decerebrate animals made spinal at the level of L1. when larger doses of eserine were used (0·6 mg.) some degree of potentiation did occur. The experiments suggest that some degree of 'long circuiting' is necessary for eserine to have its characteristic effects on the knee jerk, or at any rate for it to facilitate such responses (Fig. 3).

Effects of eserine in the decerebrate cat. Eserine injected intrathecally in doses of 0·3 0·65 mg. after a latent period of 2 3 min. (and 1 2 min. after the initial mechanical effects of the injection have subsided) causes the knee jerk to be modified in the following way (Fig. 3): the tension of the reflex contraction of the quadriceps muscle is increased in relation to the pre-injection level, or to the level at which the knee jerk settles down when the mechanical effects have passed away. This effect of eserine develops gradually, reaches its maximum intensity 5 15 min. after the injection and may last for over 15 min. The extent of the increase varies: in some preparations the tension of the knee jerk is doubled. whereas in others there is a threefold or fourfold increase. The relaxation curve of the knee jerks shows striking changes: it may be followed by small after-contractions (Fig. 3 B), which increase in number and may summate to attain a considerable level of tension which declines very gradually (Fig. 3 C); the relaxation may be interrupted by further short bursts of motor activity (Fig. 3 D). On the other hand, the greatly enhanced knee jerk contraction may be followed by an abrupt fall in tension to well below the initial base line (Fig. 3 E). This after-fall lasts for periods of 0·5 1 sec.; the curve then climbs to or above the base line (Fig. 3 E), and finally declines in the manner already described. Irregular quick or slow oscillations of the level of resting muscle tone may occur even when the taps on the patellar tendon are suspended.

These fluctuations in tone do not (for the reasons already given) represent fibrillation set up by a peripheral action of eserine which has been absorbed into the general circulation; they do not occur in the flexor muscles. Spontaneous movements, which might be called fibrillation, have never been seen in these experiments in muscles with their motor nerves cut.
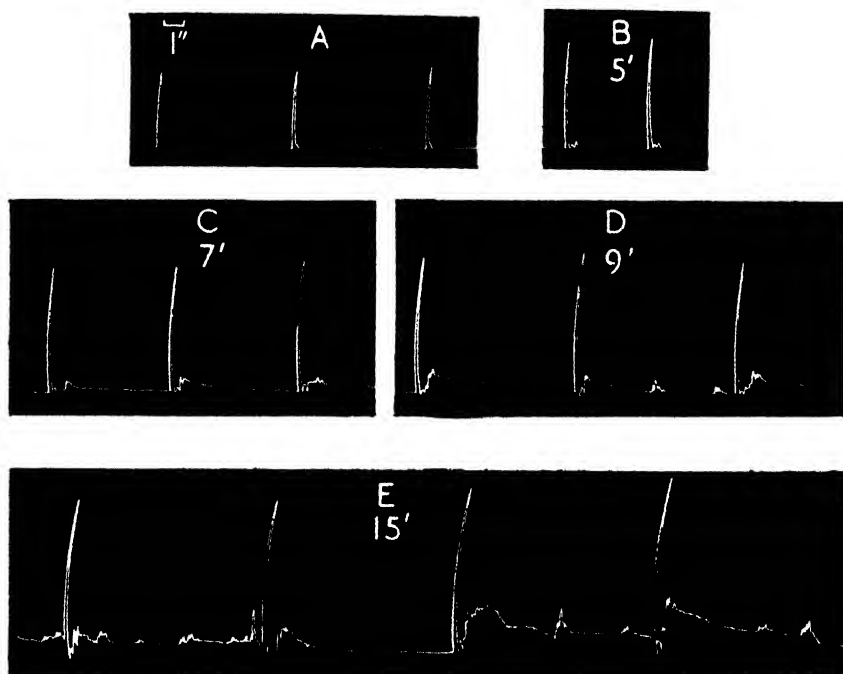
Fig. 3. Cat. 2·6 kg. Decerebrate. Spinal block at Th. 9. Knee jerk. Time scale is shown in A (fast drum). A. Before eserine. B, C, D and E were recorded 5, 7, 9 and 15 min. respectively, after the injection of eserine.

The abrupt after-fall which so commonly followed the main jerk must represent a period of central inhibition of the 'resting' reflex tone of the quadriceps muscle. Fulton & Pi-Suñer (1928) and Denny-Brown (1928) showed in action current studies that a temporary arrest of central discharge occurs after the production of the knee jerk in the decerebrate cat. Denny-Brown demonstrated that this 'silent period' was due to central inhibition reflexly set up by impulses originating in the quadriceps muscle during its contraction. Sherrington (1907) has shown that some of the afferent impulses set up by a contracting extensor muscle produce central inhibition of the motoneurone pool of that muscle. The after-fall in the experiments with eserine presumably represents a well-developed 'silent period', which might be due to a greater inhibitory volley set up by the muscle, because of its greater

tension, or to a potentiation (by the drug) of the central inhibitory effect. The observation that the after-fall may be well developed in experiments in which the enhancement of the jerk itself is small supports the latter view.

Jar reflex

In some decerebrate preparations made spinal, simultaneously with the knee jerk, a contraction of the contralateral tibialis anticus muscle was elicited during the control period. Sherrington (1898) described this reaction in decerebrate animals made spinal and showed that it was reflex in character. The sequence of events that brings about this reflex is, according to Sherrington, as follows: the tapping of the patellar tendon (say right knee) which causes the knee jerk, sets up vibrations, which are transmitted through the bones to the left leg. These vibrations stimulate the afferent nerve fibres in the posterior nerve roots corresponding with the left tibialis anticus muscle near or at the ganglion and so set up a reflex contraction. Accordingly, this 'jar reflex', as Sherrington calls it, is abolished by section of the posterior roots of the left side but not by longitudinal section of the spinal cord along the midline.

In our experience the jar reflex has been present only when the tap applied to the patellar tendon was unusually vigorous. If this reflex is initially present, it is characteristically potentiated by the intrathecal injection of eserine. Thus in one experiment the jar reflex in the control period was small and appeared irregularly. Six min. after injection of eserine, the reflex began to appear quite regularly, but was still unchanged in tension. Subsequently, the response was markedly enhanced or showed a double contraction, i.e. a small initial contraction followed by the main contraction. The effect finally passed away.

Observations on the crossed-extensor reflex

In the chloralosed cat, with spinal cord intact or transected, break-shocks, even when maximal, did not, in our experience, elicit a crossed-extensor reflex. After eserine, the crossed-extensor reflex gradually made its appearance. Initially it tended to be small and pendular in character and later showed a larger initial contraction followed by smaller secondary contractions with a gradual return to base line.

The potentiation of the crossed-extensor reflex by eserine is more strikingly shown in the decerebrate animal. The results depend on the method of recording employed, i.e. whether the tension is recorded directly from the patellar tendon or whether recording takes place from the ankle. Fig. 4 is from a decerebrate animal made spinal in which direct recording was employed. The crossed-extensor reflex (lower records) is initially small with a smooth, slightly delayed relaxation phase. Following the intrathecal injection of eserine, there were the usual transient mechanical disturbances. After 2 min. (Fig. 4 B), the reflex contraction was enhanced, and the relaxation phase showed changes

very similar to those already described for the knee jerk. Thus the initial relaxation was incomplete and was interrupted by secondary contractions.
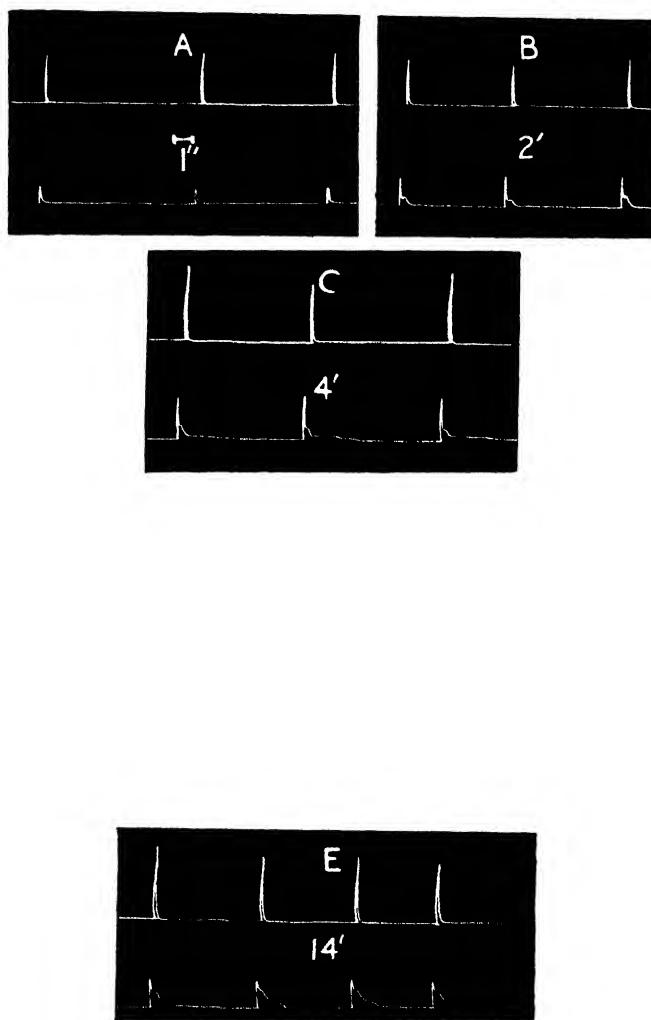
Fig. 4. Cat. 1·9 kg. Decerebrate. Spinal block at Th. 9. In each tracing the upper record is the flexor reflex recorded from left tibialis anticus; the lower record is the crossed-extensor reflex recorded from right quadriceps tendon. Time scale is shown in A (fast drum). A. Before eserine. Between A and B inject 0·3 mg. of eserine intrathecally. B, C, D and E were recorded 2, 4, 7 and 14 min. respectively after the injection of eserine.

Subsequently the initial relaxation was very slight in extent, the tension curve maintained a plateau level and then declined slowly and irregularly (Fig. 4 D). In the intervals between reflexes 'spontaneous' contractions may occur. These phenomena of the relaxation phase are to be regarded as manifestations of

different patterns of after-discharge. In Fig. 4 E (after 14 min.) though the initial reflex contraction is less potentiated, the relaxation is still prolonged and irregular.

When recording takes place from the ankle (Fig. 5), after the potentiated initial reflex contraction there is frequently (as in the case of the knee jerk) an abrupt relaxation of the quadriceps muscle with an after-fall below the base line. The tension subsequently mounts and is maintained steadily or with
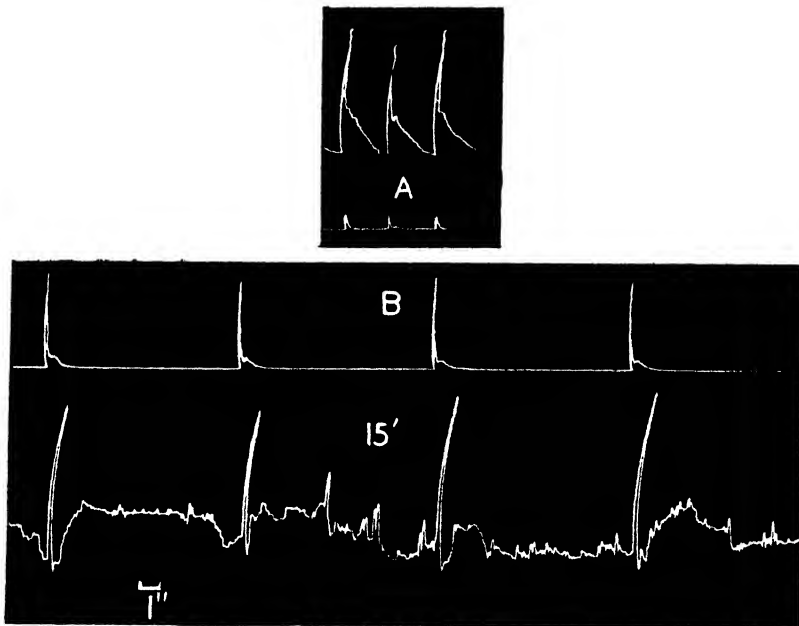
Fig. 5. Cat. 2·8 kg. Decerebrate. Spinal block at Th. 9. In each tracing the upper record is the flexor reflex recorded from left tibialis anticus; the lower record is the crossed-extensor reflex recorded from right ankle by thread running over pulley. Time scale is shown in B. A. Before eserine. Note conspicuous after-discharges of flexor reflex. B. 15 min. after injection of eserine.

irregular oscillations until an approximate return to the base line occurs (Fig. 5 B). The after-fall presumably represents a 'silent period' and can be accounted for in the same way as the after-fall of the knee jerk. The main changes in the crossed-extensor reflex represent irradiation and/or increased discharge rates of the motoneurones and prolonged after-discharge, associated sometimes with apparent intensification of some central inhibitory effects.

Observations on the flexor reflex

We have had considerable experience of the effects of intrathecally injected eserine on the flexor reflex. The outstanding feature of the results is their

variability, both in different experiments and in the course of the same experiment. In Fig. 4 the flexor reflex was enhanced. At times, though more rarely, the reflex was diminished in amplitude with decrease in the after-discharge (Fig. 5 A, B) or tended to wax and wane above and below the initial value during the course of the eserine effect. This variability of the flexor reflex was in clear contrast with the regularity of the eserine effect on extensor tone, knee jerk, crossed-extensor reflex and jar reflex.

<div align="center">DISCUSSION</div>

The effects of intrathecally injected eserine on spinal reflexes do not develop more rapidly than after intravenous injection. The effective intravenous dose in the chloralosed cat is 0·1- 0·5 mg., the drug being diluted by the whole blood volume (about 200 c.c.) and to an unknown extent by the tissue fluids before it reaches the neuraxis. The effective intrathecal dose is 0·2 0·3 mg. diluted by say 2 c.c. of cerebro-spinal fluid. If we assume that equal central effects represent the results of approximately equal concentrations of eserine in the neuraxis, then it would seem that the uptake of the drug by the neuraxis takes place much more readily from the blood than from the subarachnoid space. Difficulty in penetration through the white matter of the spinal cord may be the major source of the delay following intrathecal injection, as presumably eserine has to reach the grey matter to produce its effects.

The effects of intrathecal eserine are limited to the distal part of the neuraxis, indicating that diffusion cranially along the subarachnoid space takes place very gradually, if at all. Kremer (1942) found in man that intrathecally injected prostigmine takes hours to progress from the lumbar to the cervical region of the spinal cord.

The evidence reported here demonstrates that eserine acts directly on the central nervous system enhancing excitatory reflexes; both the immediate reaction to afferent stimulation and the after-discharge are potentiated. The drug produces its effect on crossed reflexes (crossed-extensor) and on ipsilateral reflexes (extensor tone, knee jerk and jar reflex); on reflexes employing a minimum of two neurones (knee jerk) or of three neurones (flexor reflex (irregularly)) or on multi-neuronal reflexes (crossed-extensor); on flexor responses (flexor reflex (irregularly), and jar reflex) and on extensor responses (knee jerk, crossed-extensor reflex and extensor tone).

After the completion of this work a recent paper by Wikler (1945) came into our hands. Wikler recorded the reflex efferent discharge in the ventral roots of L_7, S_1 in response to single shocks (supramaximal for A fibres) applied to various afferent nerves in cats made spinal at the first cervical segment and kept under artificial respiration and continuous ether anaesthesia. Under these conditions eserine injected intravenously (0·25 mg./kg.) enhanced the reflex discharge of two neurone arcs, while the multineurone arcs were little

affected. Morphine in preparations under nembutal was found to enhance two neurone reflex arcs and depress multineurone arcs. Eserine administered after morphine potentiated the responses from both the two neurone and the multineurone arcs. These results are in general agreement with those reported above and again emphasize the extent to which the precise experimental conditions influence the responses of the central nervous system to the effects of eserine.

The evidence reported in this paper also suggests that eserine may enhance certain central inhibitory reactions (after-fall of the knee jerk and of the crossed-extensor reflex). These last findings merit further careful consideration, in view of the previous evidence of central inhibition produced by eserine: occasional inhibition of the knee jerk in the chloralosed cat (Schweitzer & Wright, 1937 *b, c*), and in man (Kremer, 1942), occasional inhibition of knee jerk in the dog under barbital or chloralose (Merlis & Lawson, 1939), and regular inhibition of the knee jerk in the dog with isolated perfused spinal cord (Bülbring & Burn, 1941). Our own results with the flexor reflex were (as already indicated) quite irregular, both potentiation and depression being produced in different animals or at different times in the course of the same experiment.

Schweitzer, Stedman & Wright (1939) pointed out that a tertiary ammonium compound like eserine sulphate would dissociate in water into water-soluble (lipid-insoluble) ions and free base (which is lipid-soluble). They suggested that the former might be unable to penetrate the cell membrane and enter the cell, while the free base being lipid-soluble might penetrate the cell. They demonstrated that the quaternary ammonium anti-cholinesterases which give rise on dissociation only to water-soluble, lipid-insoluble ions were purely central depressant. The tertiary ammonium compounds were predominantly convulsant but tended to have a mixed central effect. The experimental evidence that has been published in recent years (including that recorded in this paper) indicates that the central inhibitory effects of eserine are of fairly common occurrence. Eserine may thus be supposed to have a dual central action, the central excitatory action generally predominating, but the inhibitory effect sometimes notably modifying or annulling it.

The study of the central action of eserine on 'pure' inhibitory reflexes may help to clarify the situation.

SUMMARY

1. The action of intrathecally injected eserine on the knee jerk, flexor reflex, crossed-extensor reflex, jar reflex and on muscle tone has been studied in the chloralosed and decerebrate cat.

2. Eserine increases the following reflex responses: knee jerk, crossed-extensor reflex, jar reflex. It increases and prolongs the after-discharges of all

these reflexes. Irradiation, facilitation and occlusion phenomena occur during the action of eserine.

3. Eserine has a variable effect on the flexor reflex, potentiating it, inhibiting it or not modifying it at all.

4. Evidence is presented of some inhibitory central effects produced or intensified by eserine, superimposed on and modifying the predominantly potentiating action of the drug.

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THE NERVOUS PATHWAYS OF INTESTINAL REFLEXES ASSOCIATED WITH NAUSEA AND VOMITING

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It has been shown (Gregory, 1946) that, when nausea and vomiting are produced in unanaesthetized dogs provided with Thiry-Vella loops of jejunum, there occur characteristic changes in intestinal tone and motility. The disappearance of these changes, following denervation of the mesenteric vascular pedicle of the loop, together with other evidence, indicates that the changes are of central reflex origin. In the experiments to be described, the efferent pathway of this reflex is shown to lie in the thoracic vagus, presumably passing thence, immediately below the diaphragm, through the upper abdominal autonomic plexuses to reach the gut in company with the nerves which arise from the autonomic plexuses. Besides the changes in tone and motility already described, nausea and vomiting also cause an increased flow of intestinal juice, which is of similar reflex origin, and for which the efferent pathway is the same.

METHODS

Six dogs provided with jejunal loops were used in these experiments; three had 2 Thiry-Vella loops each, one had 2 Thiry loops, one had 1 Thiry loop, and one had 1 Thiry-Vella loop. In preparing the Thiry loops, the cranial end of the segment of intestine was closed and the caudal end exteriorized, so that the direction of peristalsis was such as to empty the loop of its secretions. Although this choice of orientation ensures satisfactory drainage, it has the disadvantage that the balloon used for recording tone and motility is not easily kept in position. If it is intended to use such loops solely for recording motility, it is preferable to reverse the 'polarity' by closing the caudal end and exteriorizing the cranial end.

The operative preparation and method of recording tone and motility from the loops was the same as that described previously (Gregory, 1946). The flow of intestinal juice from the loops was measured by lightly covering the stomata with a weighed pad of cotton wool or square of surgical gauze backed with a piece of rubber dental dam. The complete pad weighed about 2 g. and was kept in place by means of a large pad of cotton wool and a cloth jacket worn by the animal during the experiment. These pads were changed every 15 min. and weighed immediately after removal; the increase in weight was taken to indicate the flow of juice from the loop during the preceding period. This method proved to be quite satisfactory for recording changes in the scanty flow of juice from these loops; its chief disadvantage seems to be that slight stimulation of the mucous membrane at the stomata is inevitable, and that the juice is not available for chemical examination.

Thoracic vagotomy was performed through an incision between the 6th and 7th ribs on the left side. Using ether or cyclopropane-oxygen anaesthesia, with provision for artificial respiration, the thoracic cavity was opened and the oesophagus delivered to the surface of the wound. The two or three large vagal trunks present on its surface at this point were easily identified and a portion about 1 cm. long resected from each. The oesophagus was then released and the thorax closed in the usual way. This procedure is virtually bloodless, takes only a minute or two to perform, and is far more certain in its completeness than the alternative of division of all visible fibres on the abdominal oesophagus.

Nausea and vomiting were produced by the subcutaneous injection of minimal doses of apomorphine; as in the previous experiments, a dose of 0·02-0·10 mg./kg. body weight was found to be adequate.

<div align="center">RESULTS</div>

1. Intestinal secretion during nausea and vomiting

In the fasting dog, the flow of intestinal juice from unstimulated Thiry or Thiry-Vella loops of jejunum is very slow and probably continuous, with a tendency towards rhythmical fluctuations, the cause of which remains obscure (Boldyreff, 1928; Nasset, Pierce & Murlin, 1935). Different opinions are expressed in the literature as to whether the rate of flow of juice is increased by meals; in the present experiments no significant increase was observed when the dog was fed its daily ration of raw horse-flesh during experiments in which the flow of juice was recorded.

When nausea and vomiting were produced by the subcutaneous injection of apomorphine, there occurred a conspicuous increase in the rate of flow of intestinal juice (Fig. 1) which was limited in duration to the period of other discernible effects of the drug.

Fig. 1. The effect of nausea and vomiting on the flow of intestinal juice from a Thiry-Vella loop of jejunum. At A a dose of apomorphine produced nausea alone; at B a second dose produced nausea, retching and vomiting.

Attention was first drawn to the possibility of such a secretory response by an observation that during nausea and vomiting, juice dripped from the ends of the loops at an obviously greater rate than normal; and on several subsequent occasions the fact of an increased flow in these circumstances was verified by collecting the secretion in small glass cups fastened over the ends of the loops, but not in contact with them.

This method of collection was too difficult for routine use and it was soon discarded in favour of the much more convenient and accurate procedure of collection on weighed pads; but the preliminary experiments were of value in that they excluded a possibility which would otherwise have arisen, namely,

that the increased flow of juice recorded by the pad method was merely the result of mechanical stimulation of the ends of the loops by the pads during movements of retching and vomiting. Stronger evidence against such an interpretation of the increased rate of flow is however afforded by the fact that, in two experiments, the dose of apomorphine first injected proved inadequate to provoke retching and vomiting; nausea alone resulted, without the slightest sign of a retching movement, yet there was an obvious increase in the flow of juice from the loops (Fig. 1). This flow was in fact slightly greater than that during the succeeding period of the experiment, in which retching and vomiting, in addition to nausea, were produced by a second injection of the drug.

Occasional large irregularities in the secretion curves obtained during the earlier experiments were found to be due to the expulsion from the loops of yellow pasty material. The duplex quality of the intestinal juice in the dog is well known; it consists of a colourless watery fluid, with which is mixed discrete masses or plugs of a yellow material, termed 'mucus' by the older writers (Babkin, 1928), but apparently consisting of cellular debris, cast-off mucous membrane, lipides, etc., bound together by secreted mucus. Drainage of the watery component appeared to be regular, and no indication was obtained of any appreciable accumulation of it prior to collection during an experiment. Expulsion of the solid material was, however, relatively infrequent and irregular, and it occasionally occurred during the efforts of retching and vomiting, with consequent confusion of the results. Although this material undoubtedly constitutes in a sense a part of the intestinal secretions, no satisfactory means could be found of securing its regular collection; the procedure was, therefore, adopted of washing out the loops with warm saline just before an experiment, and then allowing the loops to drain for 15-20 min. before starting collection of the intestinal juice.

This routine did not alter the general nature of the results.

The effect of denervation. In two dogs (one with 2 Thiry loops and one with 2 Thiry-Vella loops) the vascular pedicle of one loop was denervated at a second operation.

For a day or two after denervation the loops secreted continuously and copiously (though in diminishing quantity) a watery fluid, faintly alkaline and often tinged with blood, in which there was no trace of the yellow material previously mentioned, and which was without the characteristic fishy odour of normal intestinal juice. At this stage, records of tone and motility taken by the balloon method showed that the intestine was in a highly irritable state. The muscle was in a state of spasm, so that the length of the loop was found to have diminished to about one-half to one-third of its length when originally prepared: partly because of this, partly owing to a concomitant increase in propulsive motility, the recording balloon was often expelled from the caudal end of the loop within a minute or so of its introduction into the cranial end. During the succeeding 4–7 days the spontaneous secretion and motility returned to their normal state, and the yellow pasty component of the juice and characteristic smell returned. After approximately 1–2 weeks the tone and motility of the loop, the secretory response to mechanical stimulation, and the rate of spontaneous secretion, was apparently normal.

Experiments on the flow of juice during nausea and vomiting were made about 4 weeks after denervation. The secretory response previously observed under these conditions was now absent from the denervated loop, whereas a definite increase still occurred in the untouched loop of the opposite side (Table 1). This result shows that the increased flow of juice, like the tone and

TABLE 1. Intestinal secretion in a dog with 2 Thiry loops of jejunum (one denervated) during nausea and vomiting produced by apomorphine

Time (min.)	Weights of juice collected from loops by pad method	
	Left (denervated)	Right (normal)
0–15	0·11 g.	0·19 g.
15–30	0·01	0·18
30–45*	0·20	0·30
45–60	0·09	0·82
60–75	− 0·02†	0·10
75–90‡	0·04	0·13
90–105	0·05	0·48
105–120	0	0·12
120–135	− 0·01†	0·18
135–150	0·07	0·16

* Apomorphine injected at 45 min.; produced nausea, retching and vomiting at 50 min.
† Pad lost weight.
‡ Apomorphine injected at 90 min.; produced nausea, retching and vomiting at 100 min.

motility changes previously described, is of nervous reflex origin. The question then arises as to the efferent pathway concerned; and this problem was investigated by studying the effects of dividing the vagi or splanchnic nerves at their entry into the abdomen.

2. *The effect of vagal and splanchnic section on the secretory and motor responses associated with nausea and vomiting*

A. *Splanchnic section.* Although there is evidence from acute experiments that the splanchnic nerves contain fibres which may normally have an inhibitory action on the secretion of pancreatic juice (Babkin, 1928; Harper & Vass, 1941), section of these nerves under similar conditions is said to produce little or no immediate effect upon the secretion or motility of the jejunum in cats (Brunton & Pye-Smith, 1875; Florey, Wright & Jennings, 1941); and it appears to be generally agreed from examination of unanaesthetized cats and dogs at a later stage after bilateral splanchnic section that the effects produced upon the motor functions of the digestive tract are not very pronounced. Thus, Cannon (1906) found no change in the motility of the stomach and intestines of unanaesthetized cats examined by X-rays after a radio-opaque meal; others have described some acceleration of the passage of similar material, or the production of mild but not persistent diarrhoea.

In two dogs, each provided with 2 Thiry-Vella loops, the splanchnic nerves on each side were found and divided through a midline incision under ether anaesthesia at an aseptic operation. Recovery from this operation was rapid

and uneventful and no discernible changes resulted in the general health and behaviour of the animals (one showed diarrhoea for a few days afterwards). No changes were observed in the tone and motility of the loops or in the rate of the spontaneous secretion when experiments were recommenced; measurements of propulsive motility were not made.

Records of tone, motility and secretion during nausea and vomiting were begun 5 days after the above operation and continued at least once weekly for several weeks afterwards. On every occasion the characteristic motor and secretory responses previously associated with nausea and vomiting were still as pronounced as before (Figs. 2, 3).

It seems clear from these results that the splanchnic nerves do not contain fibres which are concerned in the intestinal responses described.

B. *Vagal section.* In three dogs, one of which had been subjected some weeks previously to section of the splanchnic nerves, both vagi were divided in the thorax at an aseptic operation, and, after recovery, the effects of this procedure on intestinal motility and secretion ascertained.

During a period of several weeks' observation after the first occasion on which the vagotomized dogs were offered solid food, it was clear that section of the vagi had profoundly affected oeso-phageal and gastric motor functions; and the present findings are in complete accord with the description given by Meek & Herrin (1934) of the behaviour of their dogs after thoracic vagotomy, in their study of the effects of this on gastric emptying times. At first, when the animal ate its meals, several swallowing movements were made after each mouthful, as though the food had lodged in the pharynx or oesophagus; earlier portions of the meal were frequently regurgitated and re-eaten. Saliva and mucus accumulated in the lower end of the oesophagus between meals and were regurgitated at intervals. If retching and vomiting was produced by injection of apomorphine in a fasting animal, the vomitus often contained large lumps of undigested meat eaten the previous day. None of the dogs appeared to suffer more than slight discomfort during this period of interference with gastric emptying and digestion of solid food, and they did not appreciably lose weight. In addition to raw horse-flesh they were given to drink liberal quantities of a mixture of dried full-cream milk, soya flour, cod-liver oil and water, which was seldom vomited. After a week or two, the dogs all learned to eat solid food more slowly and in smaller portions; regurgitation of food and accumulations of saliva became infrequent and the dogs returned to their former excellent condition. At this stage, their appetite was noticed to have become much larger than before operation, and it continued so for months afterwards; this change was also noted by Meek & Herrin in their dogs, and attributed by them to loss of the feeling of satiety after a meal, due to division of gastric afferent fibres in the vagi.

They also observed as did Cannon (1906) in vagotomized cats that the initial difficulty in swallowing solid food was due to paralysis of the lower end of the oesophagus, as shown by fluoroscopic examination during the eating of a radio-opaque meal.

In the present work, records of intestinal tone, motility and secretion were started 7 days after operation and were continued once or twice a week for several weeks afterwards. The tone, motility and spontaneous secretion were not significantly different from before, but the changes associated with nausea and vomiting were now completely absent (Figs. 4, 5) and there was no sign of their reappearance when the last observations were made, about 7 weeks after vagotomy.
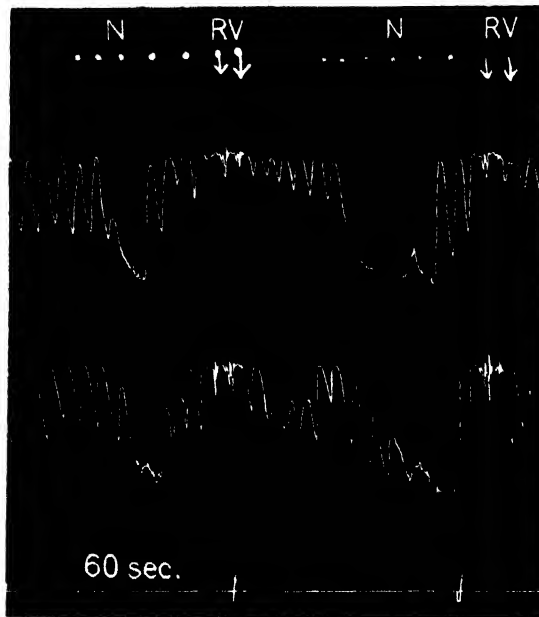
Fig. 2. The effect of nausea (*N*), retching (*R*), and vomiting (*V*) produced by apomorphine on intestinal tone and motility recorded in a dog with 2 Thiry-Vella loops of jejunum after section of the abdominal splanchnic nerves.
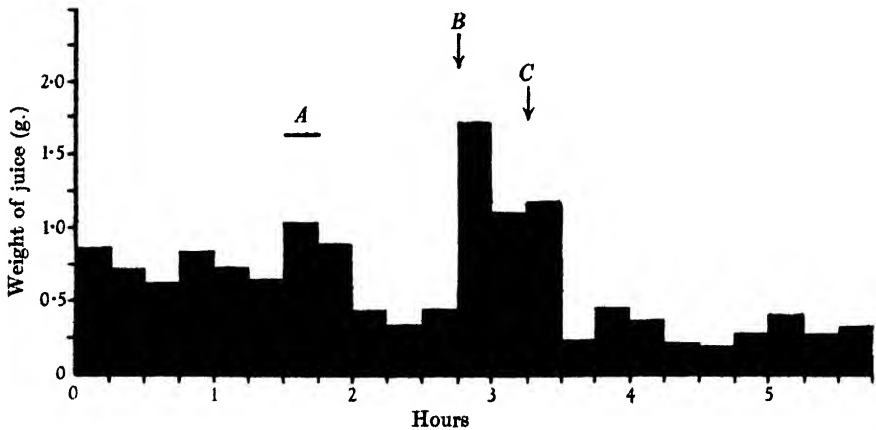
Fig. 3. The effect of nausea and vomiting on the flow of intestinal juice from a Thiry-Vella loop of jejunum after bilateral section of the splanchnic nerves in the abdomen. Nausea and vomiting were produced by injections of apomorphine at *B* and *C*; at *A* mechanical stimulation was provided by distension of the loop with the balloon used for recording tone and motility (pressure 15 cm. H_2O).

These findings appear to establish the vagi as the pathway for the fibres concerned in the changes in tone, motility and secretion of the intestinal loops
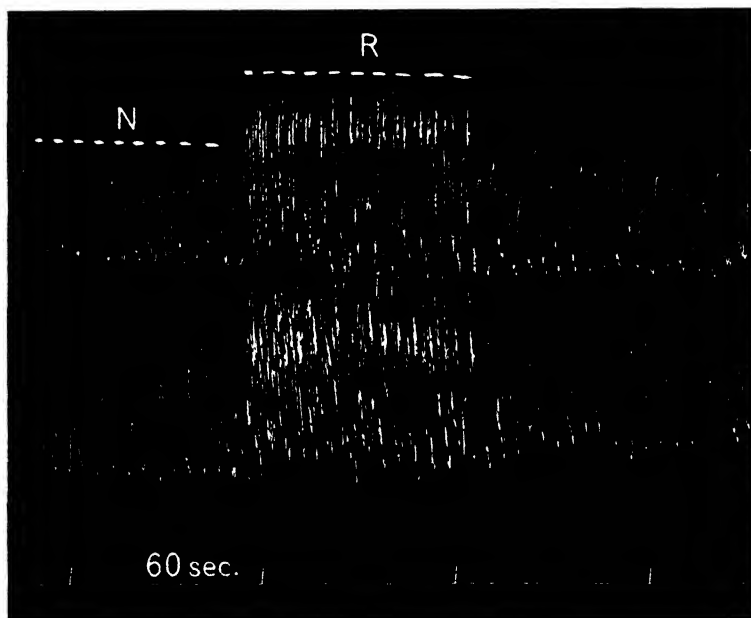
Fig. 4. The effect of nausea (*N*) and prolonged retching (*R*) produced by apomorphine on intestinal tone and motility recorded from a dog with 2 Thiry-Vella jejunal loops after thoracic vagotomy.
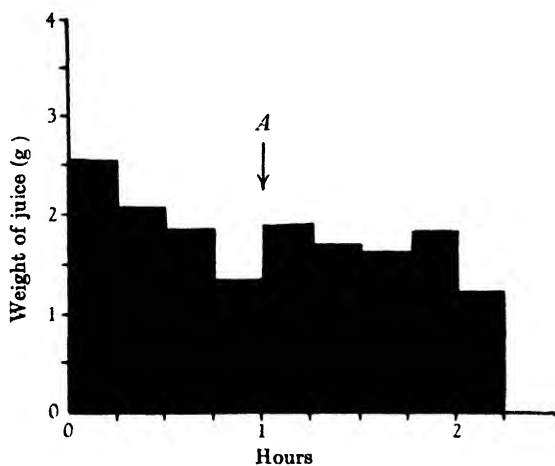
Fig. 5. The effect of nausea and vomiting produced by an injection of apomorphine at *A* on the flow of intestinal juice from a Thiry-Vella loop of jejunum after thoracic vagotomy.

during nausea and vomiting. No investigation was undertaken of the exact course of the vagal fibres from the thoracic vagal trunks to the intestinal loops;

but it is well established by the work of M'Crea (1924) and others (McSwiney, 1931) that immediately below the diaphragm the posterior vagal trunks send fibres to the coeliac ganglia. It seems highly probable that among these fibres are those concerned in the present responses, and that they run to the intestinal loops in company with the sympathetic fibres which form the main bulk of the mesenteric nerves.

It is perhaps noteworthy that in the one dog which underwent both vagotomy and splanchnicotomy, no changes were observed in the spontaneous secretion or tone and motility of the loops, comparable with those produced by denervation of the mesenteric pedicle which are referred to previously in this paper. This fact appears to have been first noted in acute experiments on cats by Brunton & Pye-Smith (1875). In order to produce the 'paralytic secretion' of intestinal juice, it is necessary to damage or remove the abdominal autonomic ganglia (Brunton & Pye-Smith, 1875) or to denervate the vascular pedicle to a loop of intestine (Moreau, 1868) or to divide all the preganglionic fibres to the abdominal ganglia (Wright, Jennings, Florey & Lium, 1940). These facts have led to the concept of the existence of a 'local centre' in the upper autonomic ganglia of the abdomen for the control of the secretion of the small intestine.

DISCUSSION

The exact nature of the intestinal movements which have been recorded in these and the previous experiments remains at present obscure. From the radiological observations of Gardiner (1928), Ingelfinger & Moss (1942) and of others on the occurrence of antiperistaltic activity in the duodenum of human subjects during nausea and vomiting, and from clinical experience that during protracted vomiting the contents of the lower parts of the digestive tract may ultimately appear in the vomitus, it appears highly probable that the intestinal activity concerned is propulsive in type and oralward in direction; the general sequence of changes recorded in these experiments by the balloon and water-manometer method, namely inhibition followed by spasm, are consistent with the occurrence of some type of peristalsis. Although the intestinal reflexes described in these experiments have been referred to as 'motor' and 'secretory' respectively. it is not intended to suggest by the use of these terms that the vagal fibres concerned are of two corresponding types. There is no evidence from the present work to suggest that such is the case, and histological investigations show that the extrinsic vagal fibres supplying the small intestine probably all end as pericellular arborizations around the Type II cells of Dogiel in the enteric plexus (Hill, 1927).

No precise information is as yet available regarding the characteristics of the motor response; but evidently the continuity of the intestinal muscle and enteric plexuses is not essential for its transmission, since it appears, in the present experiments, in loops disconnected from the rest of the gut.

SUMMARY

1. The production of nausea and vomiting in dogs provided with Thiry or Thiry-Vella loops of jejunum causes an increase in the secretion of intestinal juice.

2. This secretory response, and the changes in intestinal motility described in a previous paper, are unaltered by bilateral splanchnicotomy.

3. Both intestinal responses are abolished by thoracic vagotomy.

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THE ACTION OF SOYA-BEAN TRYPSIN INHIBITOR
AS AN ANTI-THROMBOPLASTIN
IN BLOOD COAGULATION

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It has long been held by some authorities that part, at least, of the process of blood coagulation is due to proteolysis. Nolf (1908, 1922, 1938) for instance maintains that thrombin is composed of two factors, one of which is a proteolytic enzyme. He cites the digestion of fibrin as an action of this enzyme, and points out the thrombin-like effect of certain proteolytic snake venoms. Ferguson & Erickson (1939) believe that the activation of 'thromboplastin' is the result of the proteolytic breakdown of an inert precursor, having shown that a similar activation can be produced by trypsin. Actual degradation of protein has not, apparently, been observed during natural coagulation and, if it exists, may well be so slight as to be beyond detection by the methods at present available. In this respect, the effect of known inhibitors of proteolysis on the factors concerned in coagulation might be of considerable interest, though it must be remembered that such substances may have actions other than inhibition of proteolysis.

The anticoagulant action of pancreatic trypsin inhibitor has been studied by Ferguson (1942) and Grob (1943) with somewhat discordant results. The former observed an antithrombin effect which, like that of heparin, depended upon a co-factor in the albumin fraction of the plasma. Grob, however, observed no action on thrombin, and considered that the main effect was on prothrombin or possibly on thromboplastin. Macfarlane & Pilling (1946) found that crystalline soya-bean trypsin inhibitor had an anticoagulant action similar to, but more potent than, that of the pancreatic material. There was an effect on thrombin, not greatly enhanced by the presence of the plasma proteins, but the major action seemed to be antithromboplastic, the coagulant activity of tissue extracts and of Russell's Viper Venom being inhibited. Since it was impossible to remove the inhibitor from the test systems without serious disturbance to other factors it was difficult to be certain that the delay in

coagulation was not due to inactivation of prothrombin. Grob (1943) used trypsin to remove inhibitor, but this, in view of the known coagulant properties of trypsin is quite inadmissible. This paper describes the application of a different technique to the same problem, and provides further evidence that the inhibitor acts on the complex referred to here as 'thromboplastin'.

<div align="center">

MATERIALS AND METHODS

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Buffer was prepared as follows: 1·72 g. glyoxaline and 90 ml. N/10-HCl were made up to 100 ml. with distilled water. 5 ml. of this mixture was then added to 245 ml. of 0·9 % sodium chloride solution.

Plasma was obtained from fresh human blood mixed with one-ninth of its volume of 3·8 % sodium citrate, after centrifuging at 2,500 r.p.m. for 15 min.

Thrombin prepared in dry form from human plasma was supplied by Dr R. A. Kekwick of the Lister Institute, London. Before use it was standardized in terms of the units defined by Kekwick, Mackay & Record (1946).

Fibrinogen was prepared from human plasma by the method of Milstone (1941).

Thromboplastin was made from human brain, by the method used by Aggeler, Howard, Lucia, Clark & Astafi (1946). This material was used in both the single-stage and two-stage prothrombin estimations.

Defibrinated plasma was made by diluting plasma to 1 in 4 with buffer containing 0·2 unit of thrombin per ml. After 15 min. at 37 C., the fibrin produced was removed on a glass rod and samples of the remaining fluid were tested by the addition of further fibrinogen to ensure that the added thrombin had been destroyed by the natural antithrombin of the plasma.

Heparin (British Drug Houses Ltd.) was used in the form of a powder, containing 120 i.u. mg.

Trypsin inhibitor referred to as 'the inhibitor' was a sample of re-crystallized soya-bean material prepared and kindly supplied by Dr M. Kunitz of the Rockefeller Institute.

Coagulation times were estimated in Wassermann tubes at 37 C., the end-point being clotting of sufficient firmness to prevent spilling on inversion. The recorded figures are the means, to the nearest second, of three determinations. For the two-stage prothrombin determinations, however, the pipette method of Herbert (1940) was used, and only single determinations were possible.

<div align="center">

RESULTS

Antithrombin effect of the inhibitor

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Preliminary experiments were carried out to investigate certain aspects of the antithrombin effect of the inhibitor that might bear on the later observations.

The effect of the inhibitor in relation to the rate of addition of thrombin. During experimental procedures the rate of thrombin formation may be greatly altered, or a large amount of preformed thrombin may be suddenly added to the test system. Some indication must be obtained as to whether such variations affect the antithrombin action of the inhibitor.

One volume of thrombin (12 units/ml.) was added to equal volumes of plasma and buffer (a) suddenly, and (b) over a period of 10 sec. with and without the presence of 0·2 mg./ml. of inhibitor in the buffer. The clotting times shown in Table 1 were obtained.

There is, therefore, no significant alteration in the antithrombin effect of the inhibitor with variation in rate of addition of thrombin.

TABLE 1. The clotting times of plasma and plasma-inhibitor mixture by
thrombin added (a) rapidly and (b) slowly

	Thrombin added	
	Immediately	Slowly
Plasma + buffer	11 sec.	16 sec.
Plasma + inhibitor	17 sec.	24 sec.

*The effect of varying periods of contact between the inhibitor and (a) plasma,
and (b) thrombin on the clotting times of mixtures of the three.* Plasma was mixed
with a volume of buffer containing 0·2 mg./ml. inhibitor, and incubated for
5 min. A volume of thrombin (12 units/ml.) was then added. Similarly, a
volume of the thrombin was incubated with inhibitor solution, and the mixture
was then added to a volume of plasma. The clotting times are shown in Table 2.
The antithrombin effect of the inhibitor is thus not significantly altered by
contact for 5 min. with plasma or with thrombin.

TABLE 2. The effect of prolonged contact between plasma and inhibitor, and
thrombin and inhibitor on the clotting times of mixtures of the three

	Clotting time
Plasma + inhibitor + thrombin	17 sec.
(Plasma + inhibitor for 5 min.) + thrombin	17 sec.
Plasma + (inhibitor + thrombin for 5 min.)	18 sec.

*The relative effects of the inhibitor and heparin on the clotting of (a) plasma,
(b) fibrinogen, by thrombin.* Volumes of 0·7 % fibrinogen and plasma were mixed
with equal volumes of buffer alone or buffer containing 0·003 mg./ml. of heparin,
or 0·2 mg./ml. of inhibitor. A volume of thrombin (12 units/ml.) was added to
each. Table 3 shows that, whereas the inhibitor appears to be equally active in
a fibrinogen-thrombin mixture, and a plasma-thrombin mixture, heparin loses
much of its effect on the former.

TABLE 3. The effect of inhibitor and heparin on the clotting times of plasma
and fibrinogen by thrombin

	Plasma	Fibrinogen
Buffer + thrombin	11 sec.	8 sec.
Inhibitor + thrombin	17 sec.	14 sec.
Heparin + thrombin	29 sec.	12 sec.

The effect of the inhibitor on thrombin formation

Defibrinated plasma contains all the factors necessary for coagulation except
fibrinogen. On recalcification thrombin is produced, and its concentration at any
particular moment can be indicated by the clotting time of fibrinogen added
to a sample of the mixture.

Volumes of defibrinated plasma were placed in tubes at 37° C. To the first
of these was added: (1) at zero time, a volume of 0·1 M-CaCl$_2$ containing
0·005 g./ml. thromboplastin; (2) at 10 sec. a volume of buffer; (3) at 5 min.
a second volume of buffer; and (4) at 5 min. 30 sec. a volume of 0·7 % fibrinogen.

The coagulation time was recorded. Other tubes received a volume of inhibitor solution (0·2 mg./ml.), (*a*) in place of the first volume of buffer, and (*b*) in place of the second. In succeeding tubes, heparin (0·003 mg./ml.) was substituted for inhibitor. Table 4 gives the arrangement of the experiment and the clotting times recorded.

TABLE 4. The effects of the presence of inhibitor and heparin on the formation of thrombin by recalcified defibrinated plasma

Time of addition	Agents added				
	1	2	3	4	5
0	CaCl$_2$	CaCl$_2$	CaCl$_2$	CaCl$_2$	CaCl$_2$
10 sec.	Buffer	Inhibitor	Buffer	Heparin	Buffer
5 min.	Buffer	Buffer	Inhibitor	Buffer	Heparin
5 min. 30 sec.	Fibrinogen	Fibrinogen	Fibrinogen	Fibrinogen	Fibrinogen
Clotting time (sec.)	12	111	21	41	40

It will be seen from these results that the presence of the inhibitor during the period of thrombin formation (column 2) produces a much greater effect than its addition when thrombin formation has proceeded for some minutes (column 3). Since it has already been shown (see Table 2) that prolonged contact with thrombin does not materially increase the antithrombin effect of the inhibitor, these results must be taken to indicate inhibition of the process of thrombin formation. Heparin on the other hand, has no effect on this process, its only action is on preformed thrombin.

The effect of the inhibitor on the factors concerned in thrombin formation

Three factors are supposedly concerned in the production of thrombin: ionized calcium, prothrombin and a complex known as 'thromboplastin'. Since the inhibitor cannot be removed from a prothrombin-inhibitor, or a thromboplastin-inhibitor mixture without disturbing the clotting factor concerned or destroying a hypothetical combination with inhibitor, its presence in mixtures of all three factors is unavoidable, and it may affect any of them.

The effect of the inhibitor on the activity of calcium. One volume each of plasma, thromboplastin (0·05 g./ml.) and CaCl$_2$ solutions of various concentrations were mixed and timed for coagulation. The process was then repeated, 0·02 mg./ml. inhibitor being added to the CaCl$_2$ solutions.

TABLE 5. The effect of varying calcium concentrations on the clotting time of plasma and plasma-inhibitor mixture

CaCl$_2$ concentration (M)	Clotting time (sec.)	
	Plasma	Plasma + inhibitor
0·4	57	152
0·2	19	35
0·1	15	22
0·05	12	18
0·025	14	24
0·0125	61	105

The figures in Table 5 show that the optimum calcium concentration for coagulation is the same for the plasma-inhibitor mixture as for plasma alone. It is most unlikely, therefore, that the inhibitor has any effect on the activity of calcium in this respect.

The effect of the inhibitor on prothrombin or thromboplastin. The rate of thrombin formation, in the presence of active calcium, depends upon the concentrations of prothrombin and thromboplastin. If fibrinogen is present during this process, its clotting time is determined by the rate of thrombin formation. An increase in the clotting time of such a system indicates a decrease in either prothrombin or thromboplastin, and without other evidence it cannot be determined which is affected. This point is illustrated in the following experiment.

A range of thromboplastin dilutions was made up, and a volume of each was added to an equal volume of plasma and CaCl₂ solution as for the single-stage method of prothrombin estimation of Quick (1942). The coagulation times increased progressively as the thromboplastin concentration fell, the prothrombin being constant. In Table 6, these results (column 2) are compared with those obtained when it is the prothrombin concentration that is reduced, thromboplastin being constant (column 3). The latter figures are computed from Quick's data. It will be seen that corresponding variations in either prothrombin or thromboplastin produce approximately equal effects on clotting time.

TABLE 6. The effect of variation in (*a*) thromboplastin, and (*b*) prothrombin on the clotting times obtained by the Quick (single-stage) and Herbert (two-stage) methods for estimating prothrombin

Clotting time (sec.)

Thromboplastin or prothrombin concentration (%)	Single-stage method		Two-stage method	
	Constant prothrombin (100%)	Constant thromboplastin (100%)	Constant prothrombin (100%)	Constant thromboplastin (100%)
100	13	13	16	16
50	18	16	18	26
25	27	24	18	37
12·5	34	34	20	58
6·25	44	50	21	—
3·125	55	—	22	—

Part of this experiment was repeated, using plasma to which 0·1 mg./ml. inhibitor had been added. Two concentrations of thromboplastin were used, and the following clotting times were obtained:

100% thromboplastin   58 sec.

50% thromboplastin   71 sec.

These figures indicate a delay that, from columns 2 and 3 in Table 6, might be due (*a*) to a 97% reduction of thromboplastin, or (*b*) to a 94% reduction of prothrombin, supposing that one or other of these factors had been affected.

The effect of the inhibitor on prothrombin. To distinguish between the alternatives established in the last experiment, the possible effect of the inhibitor on prothrombin was investigated more specifically by means of the two-stage method as modified by Herbert (1940). Details of this method can be found in the original publication, but the principle is as follows: Diluted plasma is mixed with thromboplastin and calcium chloride. Thrombin formation proceeds, and the maximum concentration of thrombin is determined by the periodical addition of fibrinogen to samples of the plasma mixture, and timing coagulation. Four final dilutions of plasma are used, 1 in 100, 200, 400 and 800.

Since it is the maximum concentration of thrombin that is measured, this method is relatively insensitive to variation in thromboplastin, which mainly affects the rate of thrombin formation. A series of determinations were made on a sample of plasma, using varying concentrations of thromboplastin. The results for plasma diluted 1 in 100 are given in Table 6, column 4 which can be compared with the results of variations in prothrombin produced by diluting the plasma, the thromboplastin remaining constant (column 5). It will be seen that variations in prothrombin produce a large effect, corresponding variations in thromboplastin a small one.

Determinations were then made by the same technique using the same thromboplastin solution, and the same plasma sample, but with the addition of 0·1 mg./ml. inhibitor to the latter before dilution. Table 7 shows the results.

TABLE 7. The effect of varying thromboplastin on the clotting time of diluted plasma and plasma-inhibitor mixture

| Plasma dilution | Thromboplastin (%) | Clotting time (sec.) | |
		Normal plasma	Plasma + inhibitor
1/100	100	16	18
—	20	18	19
1/200	100	26	26
	20	30	29

From these it will be seen that there is only a slight prolongation of clotting time in the case of the plasma-inhibitor mixture diluted to 1 in 100, and no delay if the dilution is 1 in 200. It can be assumed, therefore, that there is no significant inactivation of prothrombin in the diluted plasma.

Since Quick's method applied to the same plasma-inhibitor mixture suggested either a 97 % reduction of thromboplastin or a 94 % reduction in prothrombin, it may be supposed that it is the former alternative that is correct. In the two-stage method, it is unlikely that the effect on thromboplastin would be observed: (*a*) because the method is insensitive to changes in thromboplastin, and (*b*) because the inhibitor had been diluted with the plasma to 1 in 100 and 1 in 200 when the thromboplastin was added. Its final concentrations are thus 0·001 and 0·0005 mg./ml. respectively in this experiment.

DISCUSSION

Evidence has been provided suggesting that the soya-bean trypsin inhibitor exerts its major anticoagulant effect by depressing the process of thrombin formation and that this is due to inactivation of either prothrombin or thromboplastin. The two-stage method of estimating prothrombin strongly suggests that the inhibitor has no permanent action on prothrombin. A possible fallacy in this reasoning is that an inactive prothrombin-inhibitor complex may dissociate on dilution so that prothrombin activity is restored. However, taken in conjunction with previous work (Macfarlane & Pilling, 1946) which showed that a diluted inhibitor-thrombokinase (Russell's Viper Venom) mixture had lost much more of its coagulant action than could be explained by the presence of the low final concentration of inhibitor, this evidence points strongly towards a specific action by the inhibitor on thromboplastin. It must not be assumed from this conclusion alone that 'thromboplastin' therefore depends for its activity upon a proteolytic enzyme. It is of interest, however, that other evidence points to the probability that 'thromboplastin' is a complex rather than a single factor. Macfarlane, Trevan & Attwood (1941) have shown that the thromboplastic activity of Russell's Viper Venom depends on the presence of a lipoid co-factor, thus supporting the suggestion of Leathes & Mellanby (1939) that natural thromboplastin consists of an enzyme and a lipoid co-enzyme. It is possible in fact, that cephalin, supposed by various workers to represent 'pure thromboplastin', is no more than a co-factor with calcium and a proteolytic enzyme (thrombokinase), potentiating the digestion of prothrombin and consequent release of thrombin. It may be significant in this connexion that tissue extracts of high thromboplastic potency have been found to be actively fibrinolytic, and therefore, presumably, proteolytic, and that both activities are equally depressed by the inhibitor (Macfarlane & Pilling, 1946). Further investigation of 'antithromboplastins' such as the soya-bean trypsin inhibitor studied here may throw more light on the obscure prime-mover of the coagulation mechanism.

SUMMARY

1. Soya-bean trypsin inhibitor has been found to inhibit blood coagulation.

2. A minor part of this inhibition is due to a depression of thrombin action, differing from that produced by heparin.

3. The major anticoagulant effect is due to delay in thrombin formation, and, since calcium and prothrombin activity seem to be unaffected, this delay is apparently due to a depression of the activity of thromboplastin.

4. The possible significance of these findings is discussed.

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AN EXPERIMENTAL ANALYSIS OF
THE JUGULAR PULSE IN MAN

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Elucidation of the factors responsible for the waves in the human jugular pulse has been based by analogy on experimental work in animals and on the observation of synchronous events in the cycle of the human heart in health and in disease (Wiggers, 1928; Lewis, 1925; Mackenzie, 1902).

The technique of recording the jugular pulse has been adequately described by many authors: Mackenzie (1902), Morrow (1906), Bachmann (1908), Ewing (1914), Wiggers (1928), Lewis (1925), and Lamb (1930). The consensus of opinion on the factors responsible for the individual waves in the jugular pulse (Mackenzie's nomenclature) may be summarized as follows (Lewis, 1925; Wiggers, 1928):

The a wave is coincident with, and due to, atrial systole. The c wave is probably composite and includes both venous and arterial elements. The c wave is probably due, in large part, to an impulse transmitted from the underlying carotid artery and in part to closure of the tricuspid valves. The v wave is normally due to stasis alone, the blood accumulating in the right atrium and veins while the ventricle is in systole.

Mackenzie's (1902) *Study of the Pulse* was based largely on clinical material, and he assumed that the c wave was the result of the carotid pulsation.

The v wave has been the subject of disagreement (Lewis and Mackenzie). This is probably due to the fact that its time relationship to the c wave is variable. Mackenzie believed it to be of ventricular origin—thus calling it the v wave. These uncertainties justify a new experimental approach.

The procedures described here were an attempt to analyse experimentally the jugular pulse in human subjects. The object was to increase or decrease the venous components of the jugular phlebogram. It was not possible to eliminate carotid artery pulsations without obliterating those of venous origin.

METHODS

The apparatus consisted of (1) a tilting table on which the subject could be tilted into a head-up or head-down position; (2) a polygraph with jugular and carotid recording cups, the records being made on smoked paper; (3) blood pressure tourniquets connected to a pressure bottle and manometer.

The recording cups were held by hand as steadily as possible. During recording, the subjects stopped breathing at the end of a normal expiration. The venous elements in the records were simply eliminated by reduction of the venous return.

The subject was first placed in a head-down position on a tilting table, his arms being raised vertically. This drained some of the blood from the limbs. Tourniquets, previously placed in position on the four limbs, were then rapidly inflated from a pressure bottle to 160 mm. Hg, thus completely occluding the vessels of the limbs. The subject was then tilted to a head-up position at an angle of approximately 15° from the horizontal. In this position it was still possible to obtain a clear jugular pulse tracing.

While a jugular pulse record was being made in this head-up position the tourniquets were released. Evidence that, on release of the pressure in the tourniquets, an appreciable volume of blood flows into the limbs has already been given by Mackay (1943). While this volume of blood was being taken up by the limbs there was a reduction in the venous return to the heart, and consequently in the venous pressure and pulsations of the jugular vein. There was thus a reduction in the venous elements of the phlebogram.

To investigate the effects of increased venous return the subject was placed in a head-up position on a tilting table and pressure cuffs were applied to the limbs at 70 mm. Hg. These cuffs caused the veins of the limbs to fill with blood. The table was then tilted so that the subject was in a head-down position, approximately 20–25° from the horizontal. While a jugular pulse tracing was being taken, the pressure in the cuffs was released. The release of pressure allowed the pent-up blood in the limbs to flow into the great veins entering the heart. Evidence that this procedure causes an increased venous return is given by Mackay (1943).

RESULTS

Reduced venous return. In suitable subjects, results, such as are shown in the tracings of Figs. 1 and 2, were obtained. There was disappearance of the *a* wave, a reduction of the amplitude of the *c* wave and the *v* wave remained unaltered.

The tracing in Fig. 2 is divided into sections so that a clearer picture may be presented.

To clarify these changes an analysis of another tracing is shown in Fig. 3. Drawings of records, taken from the same tracing (1) before, (2) during, and (3) after, the reduction of the venous return, are superimposed. A common point of reference was taken from the apex of the corresponding carotid impulse. The point which is most common is the negative wave X'. In Fig. 3 the point corresponding to the summit of the carotid wave is marked by the curved line L. It will be noted that, when the venous return is reduced and the blood is flowing into the limbs the summit of the *c* wave corresponds to this line, whereas, under normal conditions, the line falls after the apex of the *c* wave. In other words, when the venous return is reduced, wave *c* is less in amplitude and its peak becomes coincident with that of the main carotid pulse.

In the ten subjects used for these investigations, the elimination of the venous elements of the phlebogram was successful in three. In another three

Fig. 1. Photograph of jugular pulse trace showing the effects of reduced venous return. The arrow marks the point where the limb tourniquets are released and the venous return thus reduced.
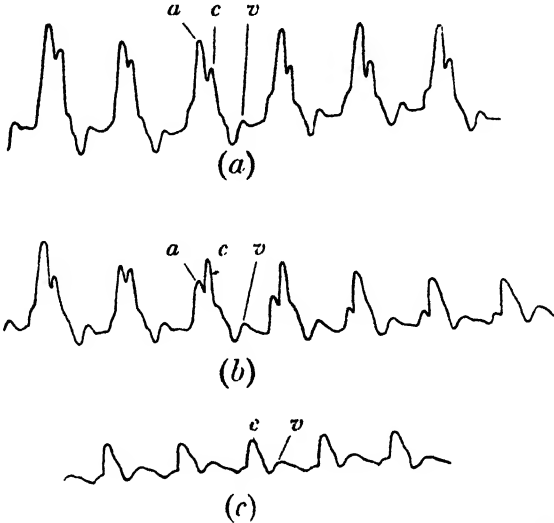
Fig. 2. Photographs of sections taken from the same jugular pulse tracing showing the effects of reduced venous return (a) before, (b) during, and (c) after reduction of the venous return. The subject was in the head-up position.
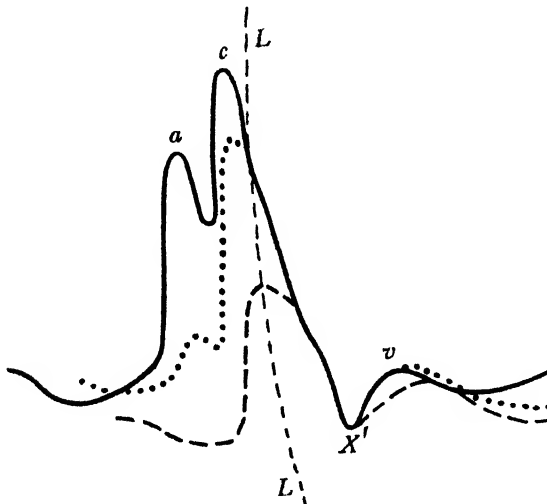
Fig. 3. Drawings made from tracings of the jugular pulse before ———; during ······; and after ———— 'occlusion' of the limbs. The broken line ———— is coincident with the apex of the corresponding carotid wave.

the elimination was incomplete, while, in the remainder, the procedures were without effect. No explanation is offered for the variability in the subjects.
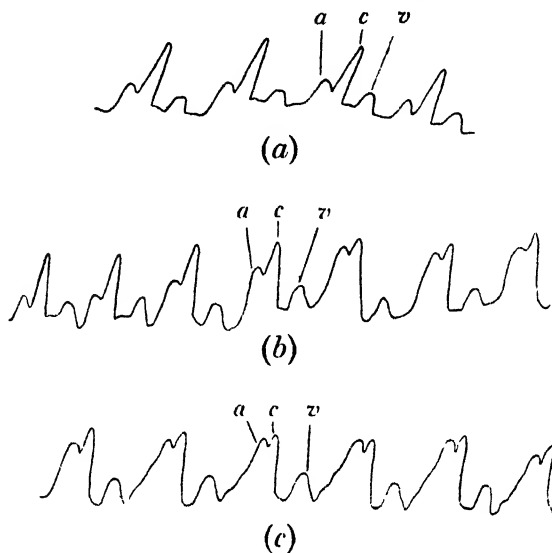
Fig. 4. Photographs from sections of a jugular pulse tracing showing the effects of increased venous return (*a*) before, (*b*) during, and (*c*) after increased venous return.
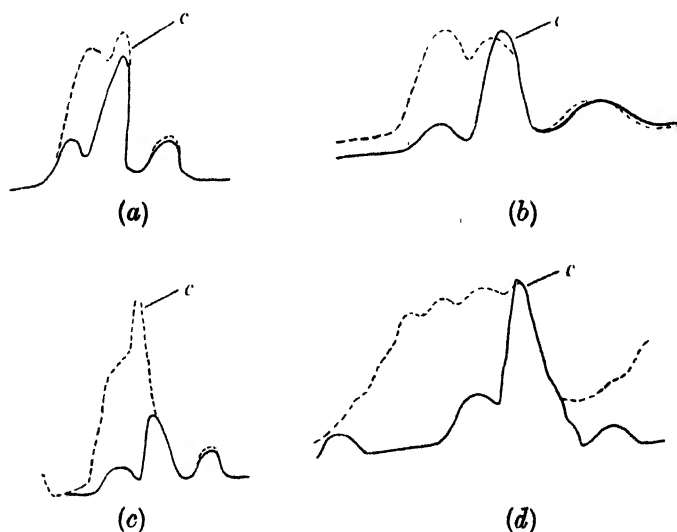
Fig. 5. The effects of increased venous return. Drawings from separate tracings in different subjects. —— Before increased venous return. – – – – During increased venous return.

In the above experiments a reduction in venous return did not appear to reduce the *v* wave, so it was decided to investigate the effects of increased venous return.

Increased venous return. Figs. 4 and 5 show typical results of the effect on the phlebogram of increased venous return.

In Fig. 4, for purposes of clarity, the tracing has been divided in a manner similar to that in Fig. 2. Here sections of a tracing, before, during, and after increased venous return are presented. In Fig. 5 are superimposed drawings taken from four separate tracings before and after increased venous return. These effects were demonstrated on seven out of the eight subjects used for this procedure.

It will be noted that, in Figs. 4 and 5, the effect of increased venous return is to increase the amplitude of the *a* wave markedly. The *c* wave rises, but not to the same extent, while (excepting 5*d*) the *v* wave is unaffected.

DISCUSSION

It may be inferred from the results obtained here, that the *a* wave is of atrial origin. This is in agreement with the present consensus of opinion. The *c* wave has both venous and arterial elements. When the venous factor is eliminated this wave is reduced in amplitude and its apex is later in time and becomes synchronous with the apex of the main wave of the carotid. The first part of the *c* wave appears to be venous in origin and the later part arterial. It has been pointed out by Bachmann (1908) that the summit of the *c* wave of the jugular phlebogram and the apex of the carotid pulse are not necessarily coincident and that *c* often precedes the apex of the carotid wave. From this he suggested that elements other than carotid pulsations enter into the formation of the *c* wave.

The uncertainties concerning the origin of the *v* wave have been noted above. The *v* wave is not influenced by varying the venous return; this does not eliminate the possibility that it is a reflexion of an intracardiac pressure wave.

SUMMARY

1. When the venous return was reduced, the *a* wave of the jugular pulse disappeared; the *c* wave was reduced, and its apex became coincident with the apex of the corresponding carotid wave; the *v* wave was unaltered.

2. When the venous return was increased, the *a* wave was markedly increased in amplitude; the *c* wave was also increased but not to the same extent, while the *v* wave was unaffected.

3. These results suggest that (1) the *a* wave is of venous origin, and (2) the *c* wave is partly venous and partly arterial in origin.

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THE OPTIMUM INTRAPULMONARY PRESSURE
IN UNDERWATER RESPIRATION

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In self-contained breathing sets, oxygen, or a mixture of oxygen and nitrogen, is rebreathed from a counter-lung (a flexible breathing bag), connected to the subject's mouth or face-mask by flexible tubing. The bag is inflated from gas cylinders carried by the subject. When these sets are worn underwater, the pressure of the water upon the chest wall is counteracted by the pressure exerted by the water on the counter-lung and thence transmitted to the gas space within the lungs. The equalization of pressure within and without the lungs can, however, only be approximate, since the chest of a diver in the erect position is exposed not to a single hydrostatic pressure, but to a pressure which increases from above downwards.

It is well known that helmet divers are sensitive to small variations in the pressure of gas respired. J. S. Haldane (1907) described in detail the sensation resulting from too low a pressure of air in the helmet, and ascribed what he observed to the rigidity of the helmet. Provision is, in fact, made for the helmet diver to adjust pressure within the helmet by means of the helmet exhaust valve. In the counter-lung breathing set, however, the diver has no delicate control of the pressure of gas in his breathing bag, apart from being able to vary the degree of filling of the bag with gas. The pressure of gas delivered to the lungs is thus fixed by the position of the bag in the water with respect to the lungs. It is evident that a breathing bag carried by an erect diver at waist level (high intrapulmonary pressure) imposes a considerable resistance to expiration, and conversely, a bag carried at head level (low intrapulmonary pressure) makes inspiration very difficult.

In the breathing sets normally in use, the counter-lung is carried either on the chest, or on the back or as a ring around the neck, and there are considerable subjective differences in comfort according to the type of bag used. The present investigation was undertaken to discover the optimum position of the breathing bag by defining the permissible limits of pressure variation for respiratory function. It soon became clear that the pressure of respired gas is discriminated

subjectively with remarkable delicacy, and for convenience the pressure of the respired gas, at which breathing was most comfortable, has been termed the 'eupnoeic pressure'. Further experiments were performed to throw light upon the cause of the eupnoeic pressure, and on the effect of deviations from it on the respiration.

Throughout this paper the pressure of the gas respired is referred to as the intrapulmonary pressure. When the intrapulmonary pressure is increased, and exceeds the eupnoeic pressure, it is referred to as high, and is plotted on graphs as positive: similarly, when pressures are less than eupnoeic they are referred to as low or negative. A low pressure therefore refers to a pressure smaller than eupnoeic, or negative, and not to one corresponding to the pressure at low level in the water.

METHODS

Subjects were investigated in the vertical and in the horizontal positions. Vertically, they were immersed at least up to the earhole in a tank 3 m. in depth. Horizontally, they rested at full length on the bottom of a tank in water at a depth of 25–30 cm. When they were supine, the back of the head, the shoulders and the buttocks touched the bottom of the tank; when prone, the face was 3–5 cm. and the upper chest 1–2 cm. from the bottom of the tank, and the lower sternum and abdomen were in contact with it.

The gas breathed was either air, oxygen or mixtures of oxygen, nitrogen and CO_2. It was always breathed through the mouth, the subject wearing a nose-clip. Work was done in the vertical tank on a pedal ergometer, specially constructed for underwater work. The subjects wore bathing trunks and goggles. The water temperature was kept at 97–100° F., except for some of the work experiments, when it was 85–90° F. The subjects were healthy males of ages 21–50 years. Two of them (C.H.L. and P.J. de C.) were Royal Naval diving instructors, experienced in the use of self-contained breathing apparatus; the remainder were laboratory workers.

Determinations of eupnoeic pressure

The principle of the method was as follows: The subject, immersed to a known depth, breathed from a Douglas Bag under pressure outside the tank; the pressure in the Douglas Bag was then changed according to the subject's signals until he had chosen the most comfortable pressure, which was noted; alternatively, the subject, while breathing air from the bag at a *constant pressure*, varied his *depth* until he was most comfortable. A typical instance was of a subject who, with his earholes 5 cm. below the surface of the water, found a bag pressure of 12·5 cm. of water to be the most comfortable; thus, the chosen (or eupnoeic pressure), relative to the earholes, was 7·5 cm.

The final form of the apparatus used in our later experiments is shown in Fig. 1.

In all determinations the subjects were instructed to 'bracket' their final choice, i.e. to try pressures too low and too high before choosing finally. As a further safeguard, the pressure at which they started was always varied considerably so that they approached their final pressure both from distinctly high and distinctly low pressures.

The eupnoeic pressure refers to the most comfortable pressure of air breathed relative to the pressure exerted by the surrounding water on the chest. It must therefore be referred to some point on the body, preferably some point whose position, in relation to the chest, is well defined, and whose distance from the surface of the water can be measured with convenience. Reference points were chosen as follows:

Vertical position. The earhole was taken as reference point. In the head-up position, the eupnoeic pressure is a pressure greater than that of the water at earhole level, and, unless explicitly stated, the eupnoeic pressure always means 'cm. below earhole'. In the head-down position, the eupnoeic pressure is again referred to the earhole, but is now less than that of the water at earhole

level; it is therefore expressed in 'cm. above the earhole', and the numerical value of eupnoeic pressure will decrease as the pressure required for comfort increases.

Horizontal position. For reference, the plane on which the subject was lying was used, i.e. a plane passing through those points of buttock and shoulder or chest and abdomen in contact with the bottom of the tank. The eupnoeic pressure was always less than the pressure of water at the depth of this plane, so that the eupnoeic pressure always refers to 'cm. above the plane on which the subject lies'.
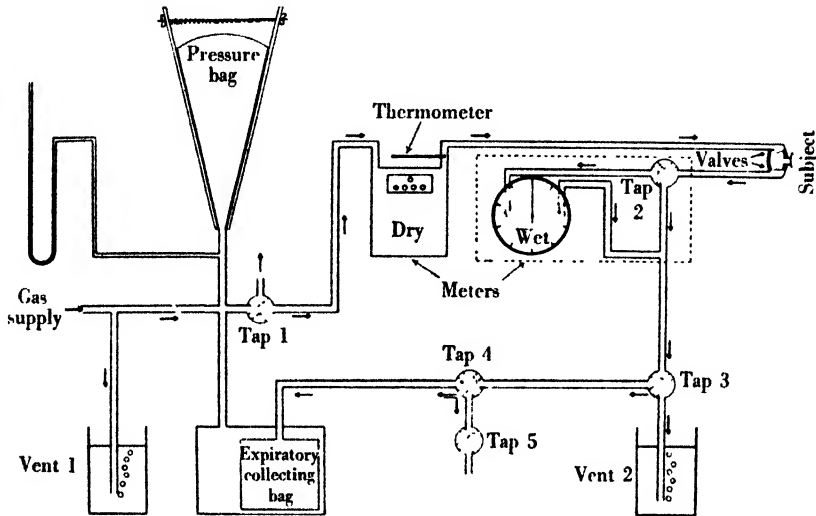
Fig. 1. Diagram of apparatus used for studying underwater respiration.

Respiration and metabolism

A diagram of the apparatus is given in Fig. 1. It consisted of (*a*) apparatus to supply gas at the required pressure; (*b*) dry meter and recording apparatus on the inspiratory side of the subject; (*c*) apparatus for collecting expired air and an expiratory vent.

(*a*) Consisted of a spring-loaded balloon-fabric pressure bag connected through a 4-way tube to a source of compressed air, to the adjustable water vent (vent 1 on Fig. 1), to the inspiratory tube, and to a pressure tank enclosing the expiratory collecting bag. The compressed gas was supplied either from cylinders or from a small portable compressor. The pressure of the gas delivered to the subject was measured on the water manometer shown, and varied by less than 1 mm. with quiet respiration, and by not more than 0·5 cm. with the largest tidal airs. The pressure was regulated by the depth of vent 1 between 0 and 30 cm. water, vent 2 being adjusted to an equivalent depth. The flow of gas was such that vent 1 bubbled continuously. Tap 1 enabled the pressure to be cut off, and the inspiratory side opened to room air.

(*b*) Consisted of a Glover dry meter, which recorded minute volumes and respiration rate on a smoked drum, using an integrating device designed by H. B. Barlow. A thermometer inserted through the tube leading from the meter recorded the temperature of the inspired gas.

(*c*) Consisted of an expiratory tube leading to tap 3, which normally directed the expired air to the expiratory water vent 2 (this consisted of a brass tube closed at the lower end, with three rows of small holes drilled in the circumference near the closed end). When samples of expired air were required, tap 3 was turned so that the air passed into the expiratory collecting bag (capacity 100 l.) enclosed in the pressure tank. This enabled expired air to be collected, without altering the respiratory pressure, for a period limited by the capacity of the collecting bag. At the end of collection, the expired air was directed again through vent 2, and the collecting bag was emptied via taps 4

and 5 to outside air, a sample being taken from the tubing between these taps. Finally, the taps were set ready for the next sampling. The samples were collected over mercury, and duplicate analyses made with the Haldane apparatus.

Calculations of the oxygen consumption and carbon dioxide production were made on the usual lines.

The accuracy of these determinations, assuming all errors to be working in the same direction, estimated by partial differentials, was $\pm 7\%$. This estimate applies to the technique only. No special precautions were taken to see that subjects were in the post-absorptive state.

Vital capacity and its components

The apparatus described in the preceding section was used, with the addition of the wet meter and 3-way tap enclosed in a dotted line in Fig. 1. This tap enabled an expiration to be delivered, when desired, through the wet meter. Inspiratory volumes were read directly from the dry meter.

In vital capacity determinations, the subject signalled just before and just after his maximal expiration, enabling the operator to pass that expiration alone through the wet meter. For determining reserve air the subject again signalled just before and after the maximal expiration, and the operator passed that expiration alone through the wet meter. For determining complemental air, the subject again signalled before and after his maximal inspiration, and the volume inspired was read on the dry meter. At least three determinations of each were made, and means were taken.

The residual air was not measured directly, but experiments were made to ascertain if it was changed by immersion. In these, the subject breathed from a Benedict spirometer while alternately out of water and immersed.

Instantaneous respiratory air flow

It was expected tha t,in view of the considerable subjective effects, the deviations in respiratory pressure studied here would affect the rate of air flow during the respiratory cycle. To test this, a sensitive compensating manometer was connected to the mouthpiece of the breathing circuit, and its deflexion was recorded photographically on moving bromide paper with a constant speed camera. By calibrating the manometer for different rates of air flow through the experimental circuit, a series of deflexion-flow calibration curves were obtained from which the peak rates of inspiratory and expiratory flow of submerged subjects during rest and work could be derived.

RESULTS

Eupnoeic pressure

The sensation by which the eupnoeic pressure is judged is complex and hard to define. Chiefly it is located in the chest, but some subjects state that the feeling of distension or otherwise in the mouth and cheeks is an appreciable factor. With low pressures, there is difficulty of inspiration and a much facilitated expiration, sometimes so much so that expiration has actually to be slowed up deliberately. With high pressures, expiration becomes hard, whereas during inspiration the lungs fill almost effortlessly. The balance struck depends on the subject: some aim for a perfect balance; others aim for a slight positive pressure so that they are certain of having no inspiratory difficulty. Subjects are unanimous that expiratory difficulty is preferable to inspiratory difficulty.

Vertical position

Resting. Determinations were made on eight subjects, the results being shown in Table 1. In addition, thirty-one determinations were made on each

of two subjects, and the distribution of the results is shown in Fig. 2*a*, *b*. It is evident that a reliable value for the eupnoeic pressure of any subject, within a few cm. of the true value, can be obtained by a few determinations.
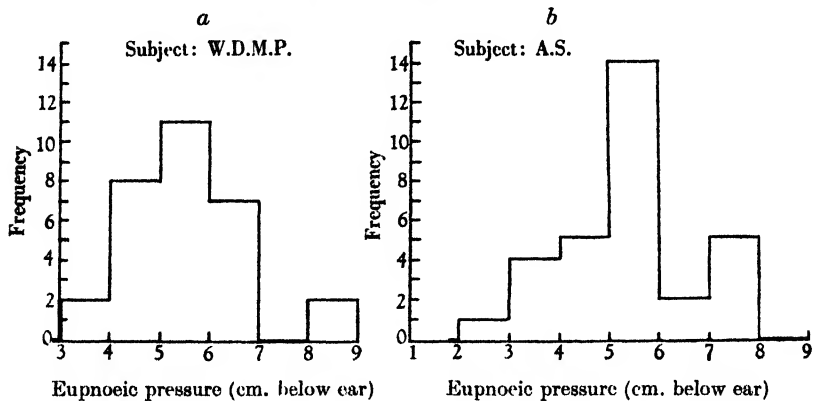
Fig. 2. Frequency distributions of values obtained by repeated measurement of the eupnoeic pressure in two subjects in erect posture.

TABLE 1. Eupnoeic pressures

Position		Subject	*E.P.* (cm. water)	Standard error of mean	No. of determinations
Erect		H.B.B.	6·1	1·05	7
Erect		H.P.M.	7·6	0·85	10
Erect		G.L.B.	6·3	3·43	3
Erect		F.C.M.	10·9	6·37	3
Erect		A.S.	5·29	0·27	31
Erect		W.P.	5·43	0·21	30
Erect		P.J. de C.	10·8	3·68	4
Erect		C.H.L.	11·5	8·13	2
		Mean	8·73 cm.	± 1·1	
Horizontal	Supine	A.S.	15·0	—	16
	Supine	P.J. de C.	11·3	—	2
	Supine	C.H.L.	14·5	—	7
	Supine	G.L.B.	15·0	—	4
	Supine	J.A.B.G.	16·1	—	3
		Mean	14·2 cm.	±3·45	
Horizontal	Prone	A.S.	10·5	—	18
	Prone	C.H.L.	12·3	—	4
	Prone	G.L.B.	10·0	—	3
	Prone ·	J.A.B.G.	13·3	—	5
		Mean	11·5 cm.	±3·46	
Horizontal on side		—	—	—	
Head straight		P.J. de C.	17·0	—	4
Head straight		C.H.L.	18·0	—	3
Head down		P.J. de C.	15·0	—	4
Head down		C.H.L.	11·0	—	4

During hyperpnoea. In two experiments, the subject rebreathed as long as he could from a 50 l. bag without removal of CO_2. In the first experiment, with a high oxygen content in the gas breathed, there was 8·3 % CO_2 in the gas at

the end of the experiment: in the second, using air, there was 7·7 % CO_2 and 11·6 % O_2 at the end. A considerable hyperpnoea resulted, during which the eupnoeic pressure rose from 5·0 to 7·3 cm. in the first experiment, and from 5·2 to 8·2 cm. in the second: a mean increase of 2·7 cm.

Another experiment was done, in which the subject indicated his preferred pressure while doing moderate work on the underwater ergometer. His eupnoeic pressure remained the same as at rest.

In further experiments, the eupnoeic pressure during 5 min. maximal exertion was investigated. As it is impossible to signal during such exertion, the subjects were asked to choose the pressure they wanted, before the work, and were asked at the end how it had suited them. There was considerable variation, but the general conclusion was that the eupnoeic pressure may increase to 15 cm. below the earhole, i.e. a pressure 5–10 cm. greater than at rest.

Effect of respiratory resistance. It appeared possible, if a resistance were introduced into the expiratory or inspiratory side of the breathing circuit, that the subject might change his eupnoeic pressure to compensate for it. Thus, with an expiratory resistance, he might lessen his eupnoeic pressure, so that the increased collapsing of his lungs in expiration would help to overcome it.

TABLE 2. Effect of respiratory resistance on eupnoeic pressure

Resistance (cm. of water at 85 l./min. air-flow)	Position of resistance	Eupnoeic pressure (cm. below ear)		
		W.D.M.P.	A.S. (1)	A.S. (2)
3·5	Absent	4·1	4·5	—
	Expiratory	4·7	2·9	—
	Inspiratory	6·6	3·9	—
	Symmetrical	—	4·9	—
17·0	Absent	5·8	4·5	5·2
	Expiratory	1·7	1·3	0·8
	Inspiratory	14·6	8·2	11·2
	Symmetrical	11·0	7·4	10·1

Table 2 shows the results obtained with two resistances, one light and one severe. They were placed so as to obstruct inspiration, expiration, or both of these. The table shows that the smaller resistance (such as occurs in most respiratory apparatus) has little effect. With the larger resistance a clear-cut result appeared: when the resistance was on the expiratory side of the circuit, a diminution in eupnoeic pressure occurred: when on the inspiratory side, or when symmetrical (i.e. inspiratory and expiratory), an increase in eupnoeic pressure occurred. It should be noted that these experiments were unpleasant because the subject felt that he was being driven into an uncomfortable compromise.

Horizontal position

Determinations of eupnoeic pressure were made on five subjects, the results being shown in Table 1. It is notable that departures from eupnoeic pressure are tolerated much better in this position: the subjective appreciation of the eupnoeic pressure nevertheless remains acute. Three positions in the horizontal plane have been investigated (supine, prone and on the side), and, as stated above, the results are expressed in terms of the surface on which the subject was lying.

Supine. The average eupnoeic pressure was $14 \cdot 2 \pm 3 \cdot 4$ cm. above the bottom of the tank.

Prone. The average eupnoeic pressure was $11 \cdot 5 \pm 3 \cdot 5$ cm. above the bottom of the tank.

On the side. The eupnoeic pressure was found to depend on whether the subject held his head straight, or allowed it to sink towards the bottom of the tank. With the head straight the average eupnoeic pressure was $17 \cdot 5$ cm. from the bottom of the tank: with the head down, the average eupnoeic pressure was 13 cm. from the bottom. The breadth of the subjects between the lateral surfaces of the deltoids was approximately 40 cm.

Vertical position head down

Determinations of eupnoeic pressure were made on two subjects while they rested with their heads on the bottom of the tank. Each wore a diving set (Davis Submarine Escape Apparatus) whose escape cock (placed at the bottom of the breathing bag) was closed during most of the time. The pressure in the bag was varied by the subject, either by releasing oxygen into the bag, or by cautiously allowing the gas in the bag to escape through the escape-cock. The pressure of the gas in the bag was measured by a mercury manometer outside the tank, connected to the bag by pressure tubing.

The subjects required pressures corresponding to that of water at levels respectively of 19 and $12 \cdot 5$ cm. above the earhole (in the inverted position).

The subjective effects of varying the respiratory pressure either side of this level were reported as similar to those in the vertical position head up.

Limits of tolerance

An attempt was made in two experiments to determine the maximum tolerable limits of pressure variation. The two subjects were diving instructors. The limits so obtained were, for the two subjects, 34 and 28 cm. greater pressure than eupnoeic, and 22 and 28 cm. less pressure than eupnoeic. The limiting factor on the side of excess pressure was inability to prevent gas escaping round the mouthpiece. With negative pressure there was pain in the lower chest and throat, and in one subject in the right shoulder (possibly 'referred' from diaphragm). The conditions were optimum for tolerating un-

pleasant pressures. Less experienced subjects, longer exposure, or work would undoubtedly have lessened the size of variation tolerable.

The reference planes mentioned so far were chosen as being the most convenient to measure underwater. Measurements of the subject showed, however, that the supra-sternal notch provides a more useful reference point, for in every position the eupnoeic pressure is at the level of the sternal notch, with one exception, viz. the vertical (head-up) position at rest, when the eupnoeic pressure is 5–10 cm. above the notch.

TABLE 3. Difference between vital capacity in air and best immersed vital capacity

Subject	Vital capacity in air minus best immersed vital capacity (l.)	Pressure when immersed (cm. below earhole)
H.B.B.	0·6	27·5
F.C.M.	0·14	28·0
A.S.	0·31	17·9
W.P.	0·68	6·4
C.H.L.	0·02	25·0
Average =	0·35	20·9

TABLE 4. Difference between vital capacity in air and vital capacity of immersed subject at eupnoeic pressure

Subject	Vital capacity in air minus vital capacity at eupnoeic pressure (l.)	Eupnoeic pressure (cm. below earhole)
H.B.B.	1·7	6
F.C.M.	0·5	12
A.S.	0·4	7
W.P.	1·0	6
C.H.L.	0·1	11
Average =	0·74	8·5

Experiments on the vital capacity

Although these experiments provide objective evidence on the effects of varying intrapulmonary pressure on respiration, much depends on the zeal of the subject; thus, the lessening of vital capacity with relatively low pressures is certainly due in part to the unpleasantness of inspiring deeply under these conditions. Subjects were instructed not to strain themselves, but to make an effort similar to that at more comfortable pressures. The results are shown in Figs. 3 and 4 and in Tables 3 and 4.

There is clearly great variation among individuals, permitting only general conclusions. Immersion vertically in the water diminished the vital capacity at all intrapulmonary pressures. This diminution is less with positive pressures and much greater with negative pressures. But even an increase in pressure sufficient to cause considerable discomfort never restored the vital capacity to a magnitude normal in air. A few experiments on the effects of high and low pressures when the subject was not immersed showed that both these diminished

the vital capacity. This suggests that high pressures underwater increase the vital capacity (relative to that at eupnoeic pressure) only by counteracting the surrounding hydrostatic pressure.
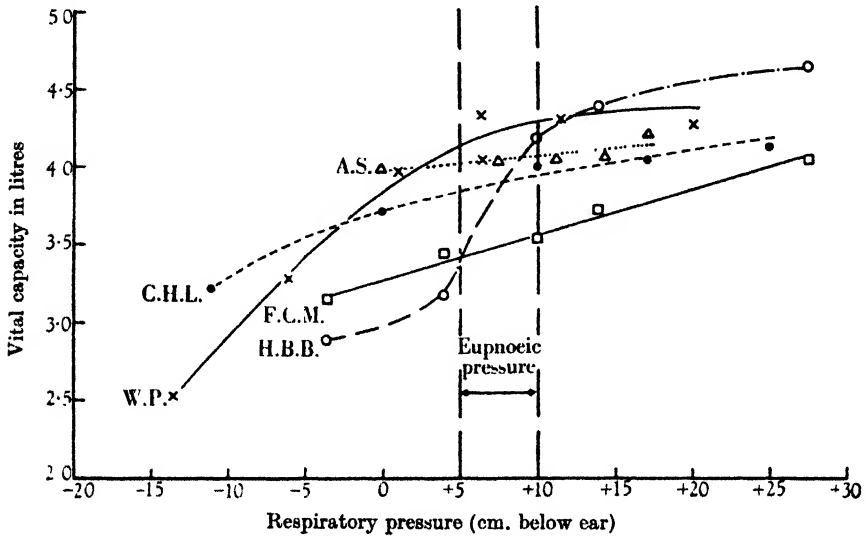
Fig. 3. Effects of varying the intrapulmonary pressure on the vital capacity when the subject is immersed vertically.
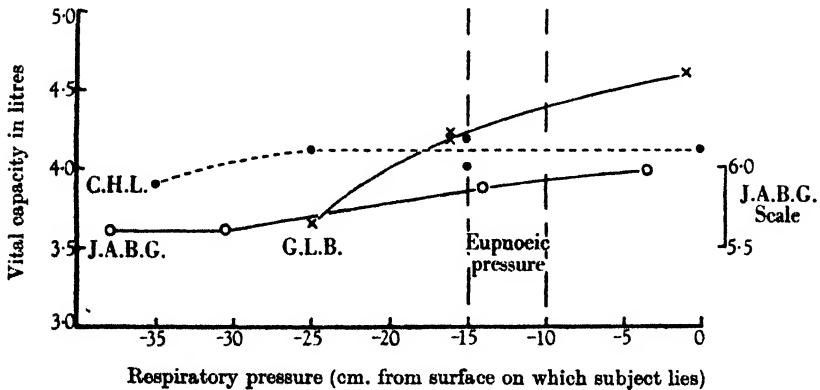
Fig. 4. Effect of varying the intrapulmonary pressure on the vital capacity when the subject is immersed horizontally.

Experiments with a horizontal position in water showed only a slight diminution in vital capacity at eupnoeic pressure, compared with that in air, and the effects of variation in pressure were small.

Complemental and reserve air. Fig. 5 shows the effect of vertical and horizontal postures and of various intrapulmonary pressures, on these components of the vital capacity. Closely similar results were obtained with other subjects.

In the horizontal position, the reserve and complemental airs are normal at eupnoeic pressure, and the tidal air occupies its normal position in the respiratory range. There is also an increase in reserve air with positive pressure—about 1 l. for 15 cm. increase of pressure. A definite, but smaller, decrease occurs with negative pressure.

In the erect position, at eupnoeic pressure, the reserve air is reduced to about 500 c.c. (range 250–700 c.c. in six subjects) and is reduced little further by negative pressures. This probably represents the lowest value it can reach within the limits of tolerable respiration. Strongly positive pressures sometimes restore it to a level normal for breathing when not immersed, but such pressures
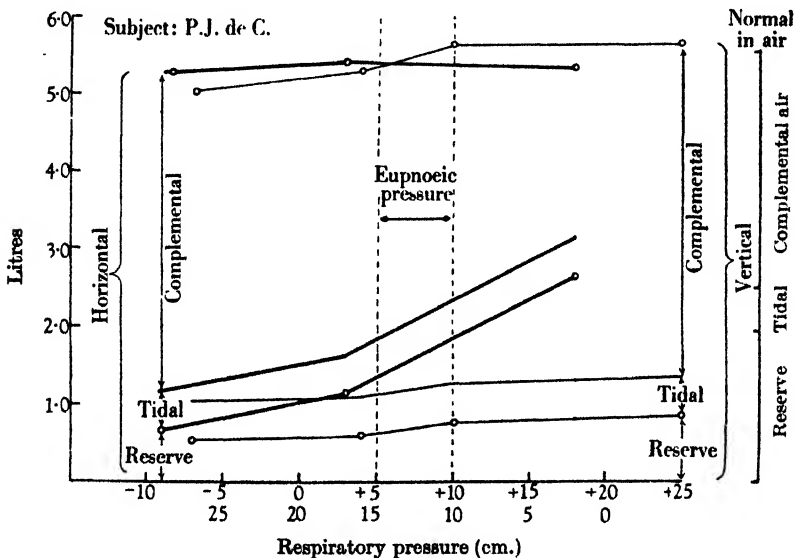
Fig. 5. Effect of varying the intrapulmonary pressure on the complemental and reserve airs. (Thick lines refer to horizontal position, with intrapulmonary pressure in cm. above supporting surface. Thin lines refer to vertical position, with intrapulmonary pressure in cm. below ear.)

are distinctly uncomfortable. Thus, for a subject immersed, the most comfortable position of the tidal air in the respiratory range is substantially nearer the limit of expiration than it is when breathing normally in air.

The changes in complemental air are all in the corresponding direction, but reflect also the changes in vital capacity. Less importance can be attached to these results, as it was not found so easy to obtain consistent maximum inspirations as to obtain consistent maximum expirations. The results provide, however, a useful verification of the results for reserve air.

Residual air. Several subjects, breathing from a Benedict spirometer at atmospheric pressure, were studied while out of water and while immersed to 3–5 cm. above the supra-sternal notch. From what had been found regarding the eupnoeic pressure, these conditions when the subject is immersed are close

to those when he is totally immersed and breathing at eupnoeic pressure. Tracings were taken of normal respiration and maximum expirations while out of water and while immersed alternately. Fig. 6 shows a typical tracing. The shift in tidal position towards expiration, resulting from immersion, is clearly seen.
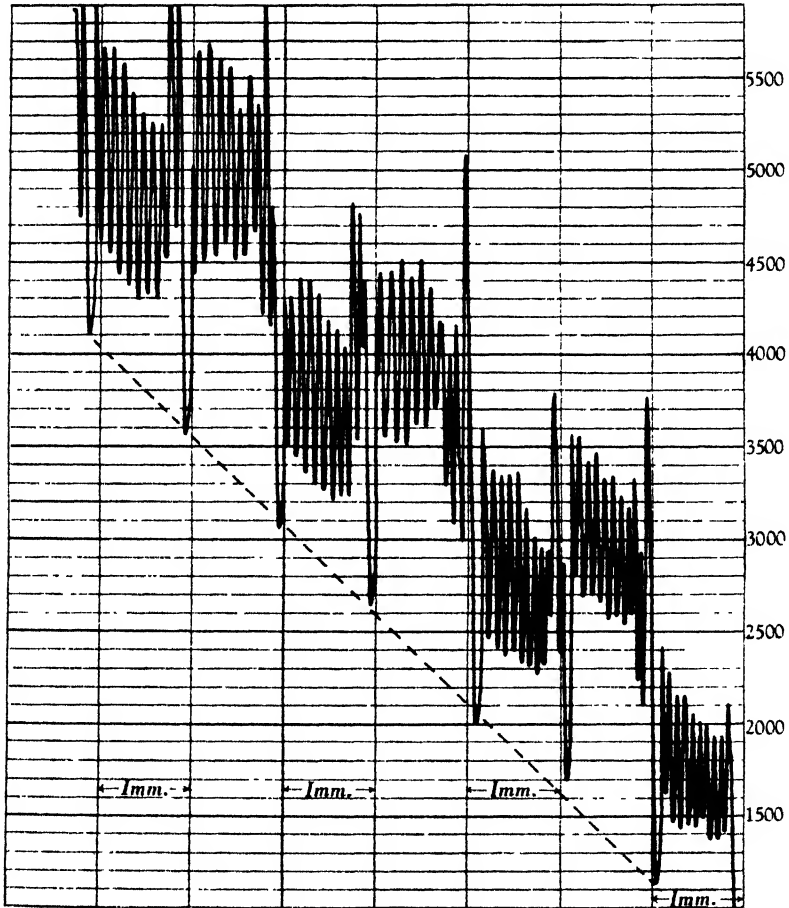
Fig. 6. Spirometer tracing of respiration and maximum expiration with the subject alternately immersed (*Imm.*), and out of water. The dotted line drawn through the ends of the maximum expirations shows that there is in this subject (C.P.) no significant change in the residual air due to immersion. (Tracing read from right to left.)

To obtain evidence whether there was also any change in residual air on immersion, a line was drawn between the lowest points of the maximum expiration while out of water. This line represents the division between reserve and residual air: on immersion, any deviation of the lowest point of maximum expiration from this line must mean a change in the residual air: if the latter

were diminished, the maximum expiration would pass below the line. Thus, although the absolute value of the residual air was not measured, any relative change in it could be detected.

In experiments on five subjects, the diminution in residual air ranged from zero to 300 c.c., averaging 150 c.c. The tracing shown in Fig. 6 is from a subject in whom immersion caused no significant decrease in residual air.

Pulmonary ventilation underwater

Previous sections have defined the eupnoeic pressure and have shown certain effects of immersion and non-eupnoeic pressures on the vital capacity and its components. It is now necessary to consider the effects of immersion and varying intrapulmonary pressure on the breathing generally.

Oxygen consumption. It is, of course, necessary to know the oxygen consumption in any experiment before the data obtained (such as minute volume or tidal air) can be compared with those under other conditions. All the data obtained have been plotted against oxygen consumption to allow for this variable.

The changes of R.Q. with increasing oxygen consumption normally found in air were also observed in these experiments. The highest oxygen consumptions recorded were about 2 l./min. Variations in intrapulmonary pressure, sufficient to produce considerable discomfort did not cause any detectable increase in oxygen consumption. Any contribution to the oxygen consumption from this cause of respiratory effort was therefore less than 50 c.c./min.

Minute volume. In Fig. 7 are plotted the minute volumes of subjects, breathing air, for various oxygen consumptions, with intrapulmonary pressures above and below eupnoeic level. Through these are drawn lines, derived from the *Handbook of Respiratory Data in Aviation* (1944), which show the average rise in minute volume with increasing oxygen consumption during work in air, and the limits which enclose 95% of their results. Two points are clear: (a) that minute volumes of the working immersed subject breathing air are closely similar to those of a subject working in air with the same oxygen consumption; (b) that variations in intrapulmonary pressure do not affect the minute volume. It should be noted, however, that one subject at rest over-ventilated with non-eupnoeic pressures, during which time his R.Q. was well over 1·0. It is probable that such individual variations would be common in subjects at rest, unused to varying pressures and at leisure to think about their breathing.

In addition, six experiments were done with subjects breathing oxygen. The minute volumes observed are not included in Fig. 7, but also fall within the same limits in all the experiments.

Tidal air. In Fig. 8 are plotted the tidal airs of two subjects (breathing air), against their oxygen consumption, with various intrapulmonary pressures.

Through the results for each subject can be drawn a line, whose position is the same whatever intrapulmonary pressure is considered. It is clear that with these subjects there was no substantial change in tidal air with changes in intrapulmonary pressure.
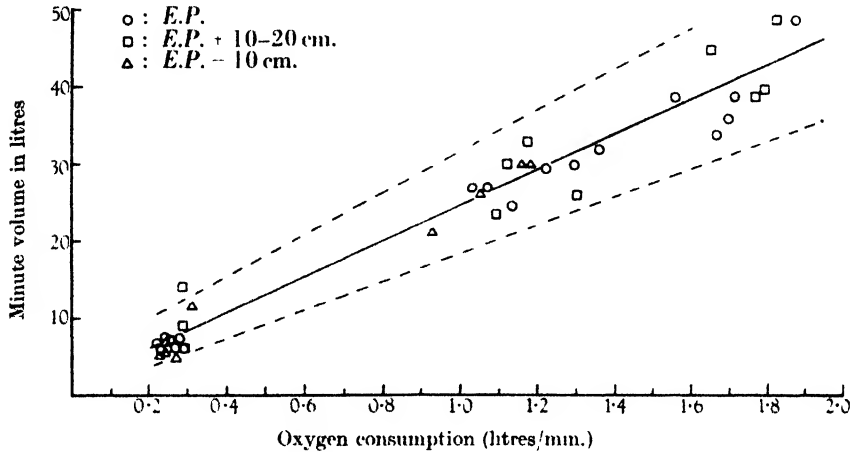
Fig. 7. Effects of varying the intrapulmonary pressure on the minute volumes at varying oxygen consumptions. *E.P.* = eupnoeic pressure.
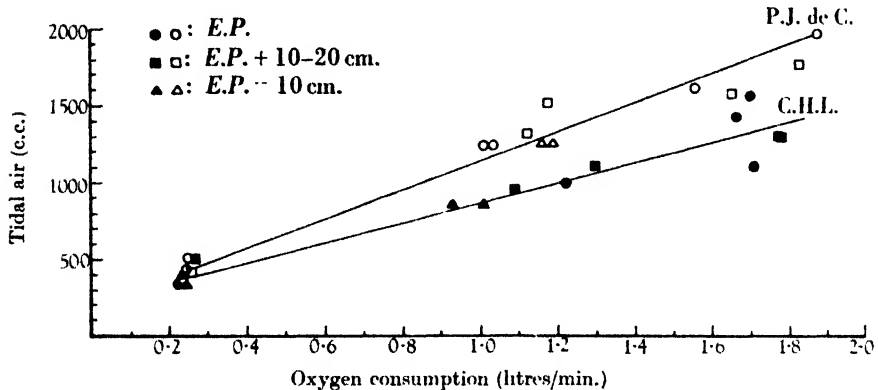
Fig. 8. Effects of varying the intrapulmonary pressure on the tidal air at varying oxygen consumptions. *E.P.* = eupnoeic pressure.

This finding means that the frequency of respiration is also unchanged with changing intrapulmonary pressure.

There were indications that the tidal air is diminished on immersion as compared to that in air. Thus, no average tidal airs greater than 2 l. were observed even with the hardest work underwater—which is certainly not the case in air. But, owing to the variability of the tidal air in and between subjects, an extended comparison between conditions in air and water would

9—2

be required. The change is evidently not a great one, since it is not enough to affect the minute volume.
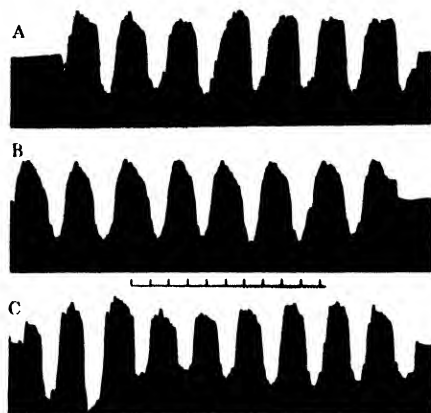
Fig. 9. Manometer records of mouthpiece pressure. Subject working underwater at 220 kg.m./min. with minute volume = 27-30 l./min. A, eupnoeic pressure; B, eupnoeic pressure + 15 cm. water; C, eupnoeic pressure − 11 cm. water. Base-line corresponds to zero flow. Downward deflexion—inspiration; upward deflexion—expiration. Time signal 1 sec.
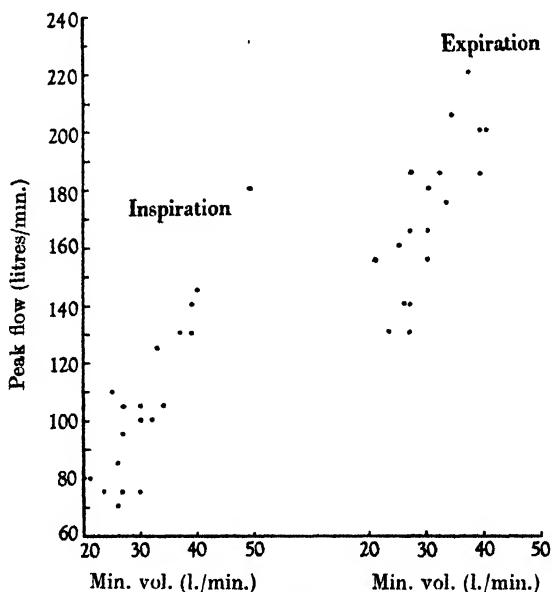
Fig. 10. Peak respiratory flow in subjects immersed in water at varying oxygen consumptions.

Response to CO_2. The minute volume, when breathing 95 % O_2 and 5 % CO_2 in the horizontal and vertical positions, was compared with that out of water when lying down. No appreciable difference was found except when negative pressures were applied; there was then a considerable accentuation of the respiratory response.

Instantaneous respiratory flow. Despite the marked subjective differences experienced with varying intrapulmonary pressures, gross differences did not appear in the manometer recordings of mouthpiece pressure: no consistent alteration was detected in duration or amplitude of either inspiratory or expiratory phases. Specimen records are shown in Fig. 9. From the records, peak flows for each phase were obtained at various minute volumes, and are shown in Fig. 10.

DISCUSSION

The eupnoeic pressure

The original aim of this investigation—the discovery of the most suitable intrapulmonary pressure underwater—has been achieved. It is possible to define this 'eupnoeic' pressure with considerable accuracy, since it is readily distinguished and falls within fairly narrow limits for the group of subjects studied, i.e. 5 -10 cm. below the ear in the upright position at rest, and at the supra-sternal notch for all other positions. Nevertheless, departures from the eupnoeic pressure, sufficient to produce discomfort, have surprisingly little effect on the character of the breathing in the steady state. They do not modify the frequency of respiration, the volume of tidal air or minute volume. They do not impair the response to CO_2. They do not, by increasing the respiratory work, cause any substantial increase in oxygen consumption or produce any change in shape or amplitude of the respiratory cycle. There is an effect on the vital capacity and its components, but the effect does not seem to be functionally important. Thus, the selection of the eupnoeic pressure rests primarily on subjective criteria, and no means were found to define its limits on a basis of objective physiological activity. That is to say, the importance of eupnoeic pressure depends on the emphasis placed on the comfort of a diver.

If a bag cannot be designed so that the pressure of air respired shall be eupnoeic in all positions, or even in the usual position of the diver, there remains the question, in which direction is deviation preferable. There is no doubt that it should be in the positive direction, for the following reasons. Positive pressures were unanimously reported as preferable to negative pressures of equal magnitude. The experiments on respiratory resistance provided a crucial test; for, where a symmetrical resistance was imposed, it was found to be treated in the same way as an inspiratory one, the expiratory element in it being relatively ignored. Positive pressures increase the vital capacity underwater. Finally, positive pressures appear to be less dangerous than negative (cf. Stigler, 1911).

It should be observed, however, that this experimental work is reassuring in that no ill-effects, other than that of discomfort, followed sustained moderate deviations from eupnoeic conditions.

These considerations are relevant to submarine escape apparatus where a low position of the venting valve, and its appreciable resistance, together with the

high rate of venting, may cause a considerable and increasing positive pressure to develop during ascent. This pressure may be substantially greater than the positive pressures investigated by us. There is no doubt that such high pressures may be very dangerous (Polack & Adams, 1932). It must be stressed, therefore, that our conclusions as to the harmlessness of positive pressures apply only to the range studied, and to steady pressures within that range.

Vital capacity and reserve air

Before considering the theory of eupnoeic pressure, it is convenient to discuss the collapse of the thorax as shown by the diminution of the vital capacity and of the reserve air, due to immersion in water. Some of this effect is due to the fact that the weight of the viscera no longer affects the respiratory apparatus. Hamilton & Mayo (1944) found a diminution of 300 c.c. in the vital capacity following immersion to the nipple line. A similar effect occurs when the horizontal posture is assumed in air; McMichael & McGibbon (1939) found an average diminution of about 500 c.c. in the vital capacity and an average reduction of the reserve air from 1·7 to 1·0 l. when a subject changed from the sitting to the lying position. The diminution in vital capacity (730 c.c.) and the reduction in the reserve air (to 500 c.c.) following immersion are, however, greater than this.

The tendency of a perfectly elastic body filled with gas and submerged is to collapse at the base and to distend at the top. The upper portion of the thoracic cage, however, is relatively indistensible, whereas the lower portion is relatively collapsible. If therefore the lungs are in communication with a gas-reservoir, so that the volume of the thorax can change without alteration in the intra-pulmonary pressure, the volume of the thorax must diminish, and to maintain it at its previous volume will require an extra inspiratory effort. It is therefore to be expected: (1) that the vital capacity will diminish on immersion, for work must be done against the water as well as against the usual resistances operating during maximal inspiration; (2) that the position of tidal respiration will shift towards expiration, for to maintain underwater the normal position at the end of an expiration will require an inspiratory effort. Unless, therefore, the end of expiration ceases to be a position of rest during immersion in water (which is not the case), we should anticipate a diminution of reserve air.

Pooling of venous blood in the thorax has been suggested as an element in the diminution in vital capacity following recumbency, and could equally well occur when the hydrostatic effects of the vertical posture are abolished by immersion in water. The results of experiments on the residual air leave this question open. It was found that residual air diminished by an amount varying from zero to 300 c.c. in various individuals. This slight diminution could be due to either or both of two causes: (*a*) the entry of an equivalent volume of blood into the thorax, the position of maximum expiration being

held unaltered by immersion; (*b*) accentuation of the position of maximum expiration on immersion, the blood content of the thorax remaining constant. (It is regarded as extremely unlikely that the position of maximum expiration should be relatively *expanded* by immersion, in view of the great diminution in reserve air.) Of these two possibilities, we favour the second, for two reasons: first, that the chest is known to be relatively collapsed, so that the effort of expiration is favoured by immersion: second, that if a shift of blood were occurring, one would expect it to occur in all subjects, rather than in only three out of five; whereas such irregularity is only to be expected where a muscular effort is concerned. Whatever the explanation, the diminution in residual air is small, and even assuming it all to be due to venous pooling, the bulk of the diminution in vital capacity found on immersion must be ascribed to other causes.

Theory of eupnoeic pressure

The main part of the following discussion refers to a diver at rest or doing light work in the vertical head-up position.

The salient point about the eupnoeic pressure is that it corresponds to a pressure of water at a level above the highest point of the lungs. It might reasonably have been supposed that it would correspond to a point somewhere about the middle of the thorax. This may be stated more rigorously as follows:

Assuming for the moment a thorax of uniform collapsibility, consider what pressure (P) should exist inside the thorax, so that the efforts of inspiration and expiration should be equal. If P = pressure of water at the level of the base of the lungs, it will be easy to inspire, but hard to expire; if P = pressure of water at the level of the top of the head, it will be hard to inspire, but easy to expire; for intermediate values of P, the resultant forces will tend to collapse the bases and expand the apices of the lungs in varying degrees. What is required is a pressure such that this collapse and expansion will balance, so that there is no *resultant* distending or compressing force; i.e. a pressure which, evenly diffused over the inside of the thorax, is just equal to the sum of the pressure gradient on the outside in an immersed subject. This pressure is familiar in mechanics as the pressure at the level of the 'centre of pressure' (or 'centroid'), a point in general slightly lower than the centre of buoyancy. There are no data as to the position of the centre of buoyancy of the chest, but it is certainly below the point half-way between supra-sternal notch and xiphoid process, and the centre of pressure is *a fortiori* at least 25 cm. below the ear, probably more.

These considerations show that an explanation is required as to why the 'expected' eupnoeic pressure is substantially greater than the experimentally determined eupnoeic pressure. It might be supposed that the basis of the sensation of eupnoeic depth is a compromise between the sensations experienced

throughout the respiratory tract, from the mouth to the base of the lungs, so that the pressure chosen is such that the sensations of inflation and deflation over the whole depth of the respiratory tract balance each other as far as possible. This implies of course, that a relatively negative pressure is tolerated by the chest and a relatively positive one by the mouth. The final pressure chosen would be expected to be about the mid-point of the whole tract, which is not far from the point determined by experiments.

There is no doubt (from subjects' reports) that mouth sensation plays a part in determining selection of eupnoeic pressure in some cases. There are, on the other hand, some subjects who definitely decide purely by thoracic sensation, and to whom this theory of sensory compromise seems inapplicable. A further difficulty is that the theory supposes a substantial negative pressure to be tolerated by the chest, as part of the compromise with the mouth; there is no such sensation experienced at eupnoeic pressure, and it is one that anybody underwater avoids, positive pressure being much preferable. It is possible that a small negative pressure is tolerated at rest, but not when working; this would contribute towards the lowering of eupnoeic pressure during hyperpnoea.

An alternative theory may be advanced, depending on the thoracic collapse discussed above. Two factors enter from this cause which will tend respectively to raise the centre of pressure and to make inspiration easier, thus lessening the expected eupnoeic pressure. The first of these is that the collapse of the base of the thorax by immersion must at once raise the centre of pressure by a small amount, simply because the dimensions of the thoracic cavity are now smaller in the downward direction.

The second factor is somewhat complicated. The normal respiratory resting position of the chest depends on the balance between the inward elastic recoil of the lungs and the outward spring of the thoracic cage (a composite force involving the ribs, diaphragm, viscera and abdominal wall). Thus an equation of forces acting in opposite directions on the thoracic wall may be written: (intrapulmonary pressure) + (intrapleural pressure) = (average external thoracic pressure) − (outward spring of thoracic cage); intrapleural pressure is, of course, · usually negative, and is equal and opposed to the thoracic outward spring. When the subject is immersed, the intrapulmonary pressure represents the eupnoeic pressure, and the average external thoracic pressure is equivalent to the pressure at the centroid. So far as immersion merely abolishes the effect of the weight of the viscera, the equality of eupnoeic pressure and centroid pressure is not disturbed, since the thoracic cage collapses a little, and intrapleural pressure is still equal and opposite to the thoracic outward spring. But the thoracic cage is collapsed further than this, as discussed above, due to its being an air-containing cavity with a relatively rigid upper part and collapsible lower part. This compression of the thorax both increases the thoracic outward

spring, and increases the intrapleural pressure (i.e. lessens its negativity), so that they cease to be equal in magnitude. Accordingly the eupnoeic pressure can no longer equal the centroid pressure but must be less, by the sum of the amounts by which the intrapleural pressure and the thoracic outward spring are each increased. The magnitude of this sum is uncertain, but can be estimated (from known variations in intrapleural pressure) as of the order of 10 cm. of water. The eupnoeic pressure then approaches the value found experimentally. (This analysis is necessarily tentative, in the absence of data on intrapleural pressure underwater, and on the magnitude of the thoracic elasticity with varying distension of the thorax.)

The above explanation is essentially in terms of the sensation of muscular effort in breathing underwater, which is desirable, since it is mostly by the balancing of inspiratory and expiratory effort that the eupnoeic choice is made, and since muscular activity is capable of the fine and continuous discrimination observed. What the ultimate sense-receptors are is not clear; probably the proprioceptors of respiratory muscle are most concerned. But other sets of receptors exist in the pulmonary field, and it is of interest that the eupnoeic level is not very far from that of the pulmonary venous pressure in the chest when not immersed; the possibility arises that afferents from pulmonary veins may transmit nerve impulses when there is deviation from eupnoeic pressure. Such nerve impulses may be responsible for the unpleasantness of negative pressures (when the veins would be distended) as compared with positive pressures (when they would be collapsed).

In positions other than the head-up. In horizontal positions, it is clear that the pressure gradient of water against the thorax is nearly abolished, so that the factors described above do not operate. We should expect the eupnoeic pressure to be about the level of the centre of pressure—i.e. a little below the centre of buoyancy—which agrees with our findings.

In the vertical, head-down position, we should expect the thorax to become distended since the distensible part of the thorax is now uppermost. This distension adds a certain volume to the upper part of the thorax, raising its centre of pressure; but at the same time the balance of forces is disturbed in the opposite direction to those previously described, so that the eupnoeic pressure must exceed the centroid pressure. Thus the eupnoeic pressure should be in the same position *on the body* as regards thoracic balance of forces, but the factor of dimension-change acts in the opposite direction. We should expect, therefore, that the eupnoeic pressure should now be nearer the supra-sternal notch and farther from the ear than it was in the erect posture. This, within the limits of the few experiments done, was found to be so.

The increase of eupnoeic pressure with hyperpnoea, in the erect position, is probably due to two factors; one, mentioned above, that a certain small negative pressure required for mouth comfort is no longer tolerated; the other,

that the subject wishing to be quite certain that he will not experience inspiratory resistance, chooses as eupnoeic pressure, a pressure with a slight expiratory resistance.

SUMMARY

1. Experiments have been made to find the most comfortable pressure of the respired air (eupnoeic pressure) when a subject is immersed. The effect of immersion on the vital capacity, on reserve, complemental and residual airs. minute volume, on tidal air and on the form of the respiratory cycle at various intrapulmonary pressures and grades of work were also studied.

2. The eupnoeic pressure is 5–10 cm. below the external auditory meatus in the erect position at rest, increasing to 10–15 cm. when there is hyperpnoea from any cause. In all positions other than the erect, eupnoeic pressure is at the level of the supra-sternal notch.

3. The vital capacity is reduced during vertical immersion, is further reduced by negative intrapulmonary pressures and is partially restored by positive pressures.

4. The reserve air is diminished during vertical immersion at eupnoeic pressure, with a corresponding increase in complemental air. In the horizontal position, at eupnoeic pressure, the volumes of reserve and of complemental air are similar to those in air. The residual air is slightly diminished by immersion vertically.

5. Deviations up to 15 cm. less, or 20 cm. greater, than eupnoeic pressure are without effect on minute volume, on tidal air and on the shape of the respiratory cycle in the steady state.

6. The origin of the eupnoeic pressure and its relation to the partial collapse of the thoracic cavity following immersion are discussed.

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THE ELECTRICAL ACTIVITY OF ISOLATED
MAMMALIAN INTESTINES

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This paper describes experiments on the origin and regulation of the pendulum movements of the intestine. Previous work (Ambache, 1946) has suggested that these movements may depend upon a nervous mechanism, acting eventually upon the musculature of the intestine by the liberation of acetylcholine, and there is already some evidence (cf. Keith, 1915) for the existence of a 'nodal' tissue in the intestine, which may control the rhythmic contractions.

The action potentials associated with pendulum movements have been recorded with a device allowing simultaneous presentation of electrical and mechanical changes on the same cathode-ray tube. The time relations of the electrical changes and the effects of suppressing the contraction upon the various phases of the action potential, suggest that the intestinal nerve net acts as a pacemaker for pendulum movements.

METHODS

The Preparation. The majority of experiments were carried out on rabbits, but a few preparations were also made from mice. All the animals were killed by concussion and bled. A coloured ligature was tied round the duodenum to identify its oral end. The small intestine was exsected and washed thoroughly with Tyrode solution, both inside and out, and a short piece of duodenum (1-2 cm.) was prepared as follows:

The gut was supported during manipulation by passing a glass rod through its lumen. Two coloured cotton threads (0·3 mm. in diameter), destined later to serve as leading-off electrodes, were stitched into the surface of the gut with a small curved cutting needle (Lane's No. 2). The stitches were placed in line on the antimesenteric border of the gut at its oral end, and the distance between them was measured. The aim was to place the stitches in the longitudinal muscle of the gut, and for this reason they were made as small and as superficial as possible. As a check on the depth of the stitches, the gut was evaginated and its interior surface was examined under a binocular dissecting microscope; in most cases the stitches were either not visible or just visible through the mucous membrane, which was itself in every case intact. Apart from this, it was difficult to assess the exact depth of the stitches, and it is probable that in some of the experiments the leads reached the plane of the circular muscle.

When this examination was completed, the intestine was re-invaginated, and after the fluid in its lumen had been gently squeezed out, it was ligated at both ends. The preparation was then connected to the apparatus which is shown in Fig. 1. It was suspended in a glass supporting

frame (*F* in Fig. 1) with the oral end upwards; the lower end was tied to a light whale-bone lever, *L*, which was hinged on to the lower end of *F*. The loose ends of the suture threads, E_1 and E_2, were then cut short (1 cm. or under) and the cut ends were slipped into the hooks, *H*, on the silver leads to the input of the pre-amplifier. A firm connexion was made by closing the hooks tightly over the threads with a pair of artery forceps. The firmness of this join (which was tested in each case) and the 'slack' in the suture threads effectively eliminated friction artefacts of the kind to which attention was drawn by Evans (1926).
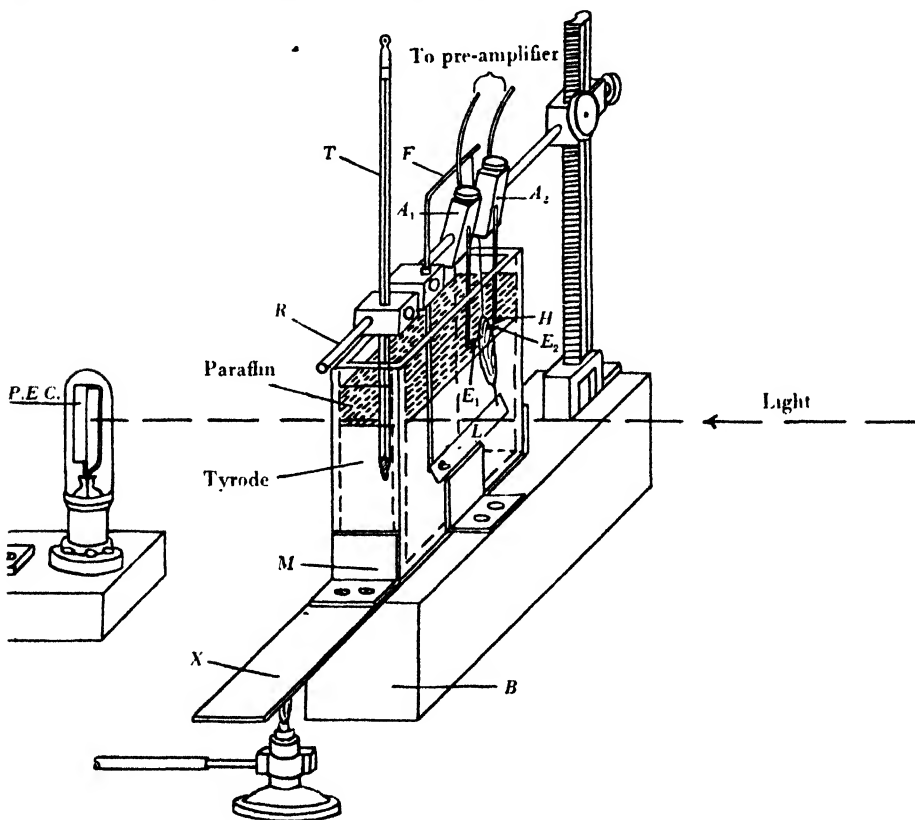
Fig. 1. Arrangement of muscle bath, electrodes and photoelectric cell. A_1, A_2, ebonite blocks; *B*, wooden block; E_1, E_2, electrode threads held in; *H*, hooked ends of silver input leads to pre-amplifier; *F*, glass frame for suspension of muscle and hinging of *L*; *L*, light whale-bone lever; *M*, metal holder for the glass bath, warmed at *X*; *P.E.C.*, photoelectric cell (Osram CMG 25); *R*, glass rod; *T*, thermometer.

The preparation was then lowered into a parallel-sided glass bath of 120 c.c. capacity filled, but for a layer of medicinal liquid paraffin on top, with unaerated magnesium-free Tyrode solution. This bath was encased in a metal holder, *M*, which was mounted on a wooden block, *B*, and protruded over one edge of this block at *X*, where it was heated by a small gas flame till the temperature of the bath was between 32 and 40° C. (35–39° C. when records were taken). When electrical records were being made, the preparation was raised so that the electrodes lay in the layer of paraffin, but care was taken to avoid too long immersion of the gut in paraffin as this led to inactivity of the tissue; at the same time, the lever, *L*, was brought into the edge of a light beam previously directed on to the photoelectric cell.

The separation of the electrodes. During the actual experiment, the distance between the electrode sutures was liable to depend upon the tone of the preparation and the load on the muscle, both of which were variable. It has, therefore, been more convenient, in order to standardize the conditions of this measurement, to quote, in the legends and in the results, the distances measured at room temperature when the gut was fully relaxed.

Recording system. The recording system owes much to the kindness of Mr E. S. McCallister of the Mullard Wireless Service Co., and is described by him (1947) elsewhere in greater detail. The apparatus consisted of a Mullard cathode-ray oscillograph (type E800) modified to provide two separate channels of amplification, one for the photoelectric impulses produced by the mechanical contractions, and the other for the action potentials.

Switching device. The outputs from the two amplifiers were fed into a mechanical vibrator (Wright and Weaire, type ACD/6), which connected the vertical deflector plates of the cathode-ray tubes alternately to each amplifier at a frequency of 100 cyc./sec. Owing to this 'chopping' frequency being high compared with the frequency of the two phenomena studied, a faithful reproduction of them both appeared simultaneously on the screen of the cathode-ray tube. To provide a separate base-line for each phenomenon a variable bias voltage was applied to the output of one amplifier relative to the other. A continuous record of the two phenomena was taken with a moving-film camera.

Characteristics of the pre-amplifier. Records were taken between a caudally situated earthed electrode and another, proximal electrode connected to the grid of the first valve of the pre-amplifier. In all records shown, an upward deflexion indicates negativity of the grid electrode. The high input impedance ($2\,\mu F. + 3$ megohms) permitted the use of 'polarizable' metallic electrodes (cf. Hodgkin, 1937). The inter-valve coupling time constants ($1.5\,\mu F. - 3$ megohms) were adequate for the requirements of this research, being sufficiently large compared with the duration of the action potentials studied.

The overall sensitivity of this channel was approximately $70\,\mu V.$ (d.c.)/cm. deflection on the cathode-ray tube. The noise level was 15–$20\,\mu V.$ Small fluctuations of the base-line were effectively prevented by the second valve in the pre-amplifier which acts as a stabilizing device.

Interpretation of the photoelectric record. The movements of the muscle lever in the beam of light produce a voltage variation which is amplified in the second channel. Because of the condenser coupling between the valves, it is evident that long-lasting changes of tone will not be faithfully reproduced by this amplifier. Thus the records provide no indication of the tone level of the preparation. This is a drawback which did not matter in the present experiments, the aim being to show the time relation of the action potentials to the onset of each rhythmic contraction.

Despite the large coupling time constants, a certain amount of distortion of the contraction curve is apparent in some of the photoelectric records. Thus, when the interval of relaxation between successive pendulum movements is prolonged (Fig. 2c), there is a slow upward deflexion, starting some 2–3 sec. before the true contraction of the muscle. This is probably due to the condenser decay of the steady p.d. from the photoelectric cell during the 3–4 sec. of the relaxation phase. The true onset of contraction is indicated in the records by the sharp upward inflexion in this curve, which is coincident with the beginning of each pendulum movement.

The tracings were timed at the end of each experiment by interrupting the light beam at fixed intervals by means of a clock.

RESULTS

The electrical activity attending pendulum movements

The experiments were carried out without oxygenation of the bath and with the lumen of the gut completely empty. This procedure was calculated to abolish active peristalsis without affecting the pendulum movements (Ambache, 1946); in fact, peristaltic contractions were never observed.

The records obtained under these conditions reveal the existence of

rhythmically recurring electrical changes associated with each pendulum movement. With the preparation as short as possible (1 cm.) and the distance between the electrodes small, the potential changes observed are of two kinds, each with its own temporal characteristics. There is first (Fig. 2) a slow diphasic wave, 'A', which *precedes* the contraction; in Fig. 2*b* the time interval
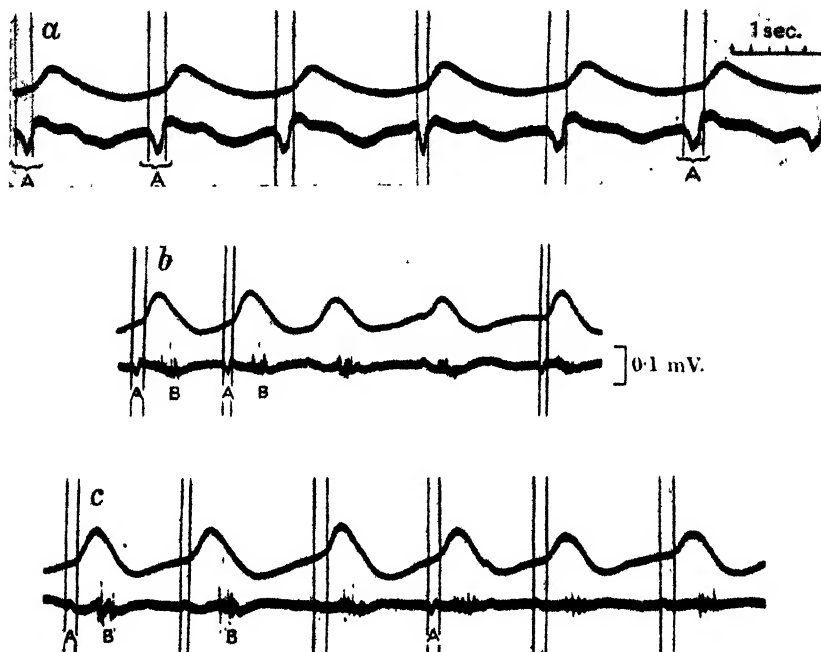
Fig. 2. Simultaneous records of the rhythmic contractions (top line: photoelectric record) and the action potentials (bottom line) of a preparation (1 cm. long in the relaxed state) of fresh rabbit's duodenum. This and all subsequent records should be read from left to right; time, 1 sec. Calibration: 0·1mV. (d.c.). (*a*) Leads αγ (1 cm. apart). Diphasic 'A' waves are seen preceding each contraction. An upward deflexion indicates negativity of the grid electrode (oral end of the muscle) in this and all subsequent records. Paired vertical lines have been drawn through (*a*) the onset of the 'A' waves, and (*b*) the beginning of each contraction, to show the time lag between the two phenomena. (*b*) and (*c*) Leads αβ (1 mm. apart); showing the polyphasic response 'B' which follows 'A'. 'B' occurs during the contraction.

between these two phenomena is 0·5 -0·75 sec. This time interval is also found when the contractions are recorded by a direct optical method. For this purpose, a ground-glass screen was mounted above the face of the cathode-ray tube, and a beam of light was reflected from an optical lever (in air) on to this screen. The alignment of the two 'spots' was carefully checked with a plumb-line at the time of the experiment and, later, on the photographs. This experiment also showed that the damping effect of the bath fluid on the lever (in the photoelectric method) was negligible.

The 'A' wave is followed by a faster, polyphasic response, 'B', which occurs *during* the contraction. In Figs. 2*b, c*, the distinction between 'A' and 'B' is quite clear, but in other records it is sometimes obscured by the merging of 'B' into 'A'. By various means, which are described below, it has been possible to obtain the first wave, 'A', without the other (e.g. in Fig. 2*a*), and for this and a number of other reasons, given subsequently, it is possible to interpret these two types of potential recorded as manifesting the activity of two different tissue components within the gut.

Berkson, Baldes & Alvarez (1932) have reported the occasional occurrence of contractions without any electrical change. In the present experiments this has been noticed in fresh preparations only twice; it appears to happen when that part of the muscle which is immediately under the electrodes, i.e. the top end of the gut, is left for too long in the paraffin layer. To avoid this local failure, the records were only taken immediately after the muscle was raised into the paraffin.

Effect of electrode separation

The following experiment was designed to show the effect of varying the distance between the electrodes on the type of activity recorded. A third silver electrode was included in the bath, and the gut was prepared with three leading-off sutures: one (α) at the top end of the muscle; the second (β) 1 mm. below α, and the third (γ) at the bottom end of the gut, 1 cm. below α. The muscle was permanently connected to the grid of the input through α, but it could be earthed at will through β (arrangement $\alpha\beta$; electrodes 1 mm. apart) or through γ (arrangement $\alpha\gamma$; electrodes 1 cm. apart).

Leads $\alpha\beta$ (1 mm.). An inspection of the records shows that the waves 'A' and 'B' are invariably present (Fig. 2, *b, c*); their timing with respect to the mechanical contraction needs no further description. The amplitude of 'A' is usually 50 μV. from peak to peak, but the amplitude of the individual component waves in 'B' varies from one wave to the next, and often from contraction to contraction, ranging between 50 and 200μV. There appears to be a general tendency for the whole 'B' complex to decrease as the contractions get smaller, when, for instance, the muscle is left too long in the paraffin layer. In certain cases, where the component waves in 'B' are sufficiently spread out, one can make out their diphasic nature (Fig. 2*c*).

Leads $\alpha\gamma$ (1 cm.). In these records the 'A' wave is still regularly present and always precedes the onset of the mechanical contraction, often by as much as 1 sec. (Fig. 2*a*), but there has been a noticeable change in the shape of this wave, which shows considerable broadening. Thus the distance between the peaks of this diphasic wave (wherever these can be made out distinctly) shows a four- or fivefold increase. There has also been an increase in its amplitude (now 100–110μV.), but it is difficult to know whether this is merely due to a difference

in the depth of the electrode sutures at β and γ or to other causes. On the other hand, the broadening of 'A' indicates clearly an increase in the interelectrode conduction time of this response, as the electrodes are separated.

The other noticeable change in the $\alpha\gamma$ records is the frequent absence or attenuation of the polyphasic response 'B'. When present (in only sixteen out of ninety recorded contractions, in the experiments of Figs. 2 and 4), the average amplitude of this response is reduced, but its temporal characteristics are unaltered (see also Fig. 3). In another experiment the amplitude of the 'B' response was quite considerable in the $\alpha\beta$ records (Fig. 5b, c). Despite a certain amount of attenuation, these spikes are still clearly visible in the $\alpha\gamma$ records from the same preparation (Fig. 3).
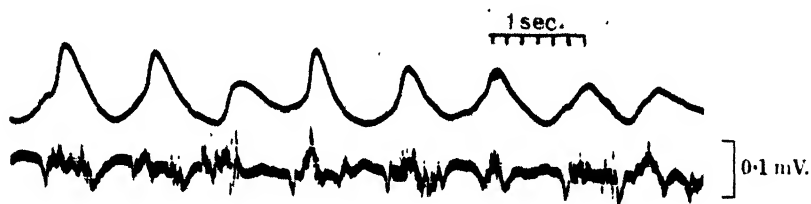
Fig. 3. Effect of electrode separation on the 'B' response. $\alpha\gamma$ records (electrodes 1 cm. apart) from a rabbit's duodenum, showing attenuation but no significant broadening of the individual waves in 'B'; for comparison, see the $\alpha\beta$ records (electrodes 1 mm. apart) in Fig. 5b and c, which were taken from the same preparation. Nicotine was present in the bath in a concentration of 1 in 10^4.

Spontaneous reversal of conduction of the 'A' wave

In their original experiments on pendulum movements, Bayliss & Starling (1899) noticed that the rhythmic contraction waves in the intestine 'pass as often in one direction as in the other', although there is 'a preponderance of descending contractions'. The electrical records sometimes show a corresponding change-over in the direction of conduction of the 'A' wave, occurring spontaneously in the middle of an experiment. The two phenomena appear to be related.

If we consider negativity as a sign of excitation, we see that throughout the experiment of Figs. 2 and 4a, the contractions are initiated by an excitation wave which passes the earth electrode first and then the grid electrode, and is in fact travelling, in this case, from the caudal end of the preparation upwards. Towards the end of this experiment a few contractions were recorded which were initiated by an excitation wave travelling in the opposite direction. This wave, the mirror image of 'A', has been called 'C'. This is shown in Fig. 4b, where the 'C' wave has for two beats superseded and replaced the 'A' wave, the excitation

travelling downwards (from grid to earth); but at the third beat excitation from below preponderates again and 'A' is reinstated. This phenomenon has been recorded in a number of experiments. The time interval between the conducted electrical variations ('A' or 'C') and the onset of contraction (or its peak, in other experiments) is independent of the direction of the conduction. The 'C' wave is also seen, sometimes, just after 'A', as, for example, in Fig. 4a, where it would seem that the preparation is discharging at both ends, excitation from below occurring first. The possible effects of such a double excitation is shown in another experiment in which the length (4 cm.) of the preparation
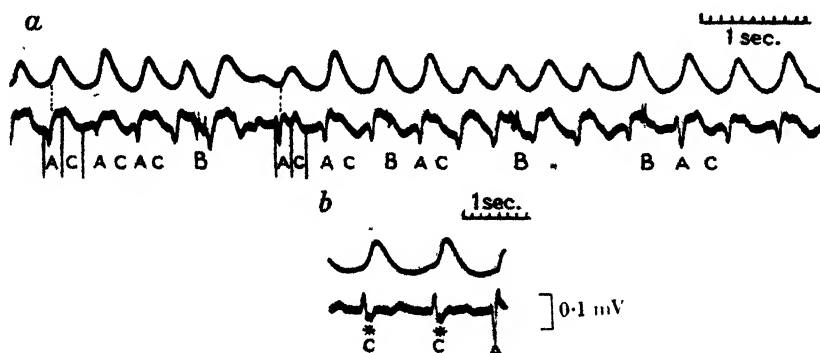
Fig. 4. (a) Continuation of the same experiment as Fig. 2. Leads αγ (1 cm. apart) showing 'A' and 'C' waves, and occasional 'B' responses. The continuous vertical lines delimit the 'A' and 'C' waves; the dotted vertical line shows the onset of contraction. For explanation see text. (b) Later. Spontaneous reversal, for two 'beats' (marked with asterisks) of the electrical disturbance which precedes contraction, producing a 'C' wave (see text); conduction is now from grid to earth, i.e. from the oral end downwards. The third 'beat' is preceded by an 'A' wave; conduction is again from the earth to the grid electrode, i.e. from the caudal end upwards. The time interval between the electrical change and the onset of contraction is independent of the direction of conduction.

was greater than usual. The photoelectric records from this preparation (Fig. 6e) showed a 'step' in each contraction, the muscle appearing to contract in two halves. The electrical change which was recorded at the top end of the muscle just preceded the second half of the contraction.

Persistence of both types of potential after nicotine

A few experiments were carried out in the presence of nicotine. A concentration of 1 in 10^4 was used, as previous experiments had shown that this was more than sufficient to paralyse the ganglion cells in the rabbit's gut. The results of two experiments are shown in Fig. 5. In the first (Fig. 5a), the 'A' waves were well marked and continued after the introduction of nicotine, but the 'B' waves were diminished or absent, possibly through depression of the muscles fibres in the interpolar field; in fact, such a concentration of nicotine

has in previous experiments sometimes produced a noticeable depression in the size of pendulum movements. In the second experiment polyphasic 'B' responses were seen both before (Fig. 5b) and after (Fig. 5c) nicotine. This experiment was remarkable for the irregularity of the contraction record, and for the merging of the two components in the electrical records. The two experiments taken together show that both the 'A' and 'B' waves can persist in the presence of high concentrations of nicotine (see also Fig. 3).
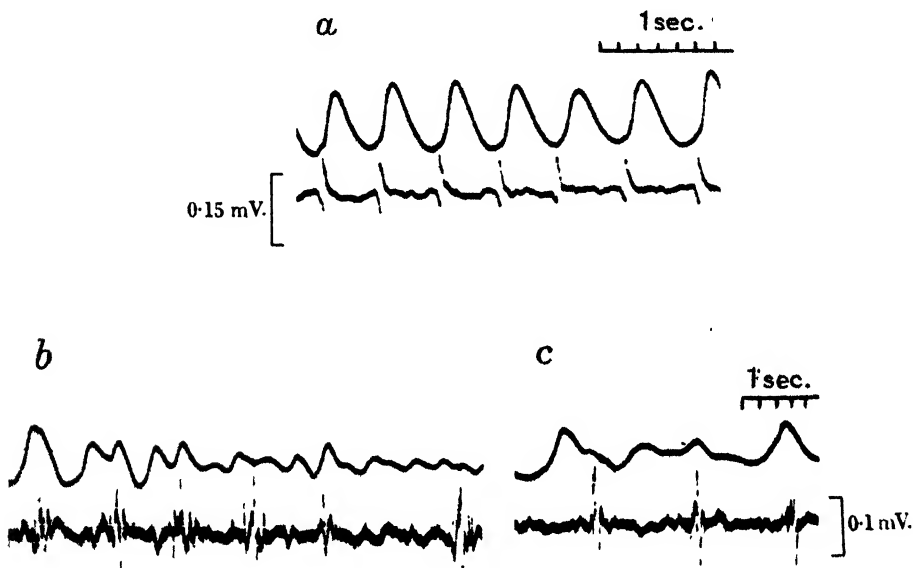
Fig. 5. Effect of nicotine on the duodenum of two rabbits. (a) Persistence of 'A' waves in the presence of one part in 10,000 of nicotine (electrodes 1 mm. apart). (b) and (c) From another preparation (electrodes 1 mm. apart). (b) Initial activity consisting mainly of polyphasic 'B' responses merging into the 'A' waves. (c) Persistence of the 'B' response in the presence of nicotine (10^{-4}).

Electrical activity in the absence of contraction

Several different ways have been employed of arresting the movements of the gut. With the exception of chloroform they all produce the same modification in the recorded response.

(a) *Calcium*. The suppression of pendulum movements by an excess of calcium ions has been shown, previously, to be attributable to an inhibition of acetylcholine release at the nerve endings (Ambache, 1946). It was of interest, therefore, to see what changes were produced by this substance in the electrical response of the gut. The more sensitive method of recording the contractions photoelectrically shows that, even with concentrations of $CaCl_2$ as high as

0·4 % (20 × normal), pendulum movements may persist, although they are now slightly irregular and very minute (Fig. 6*d*). With the reduction in size of
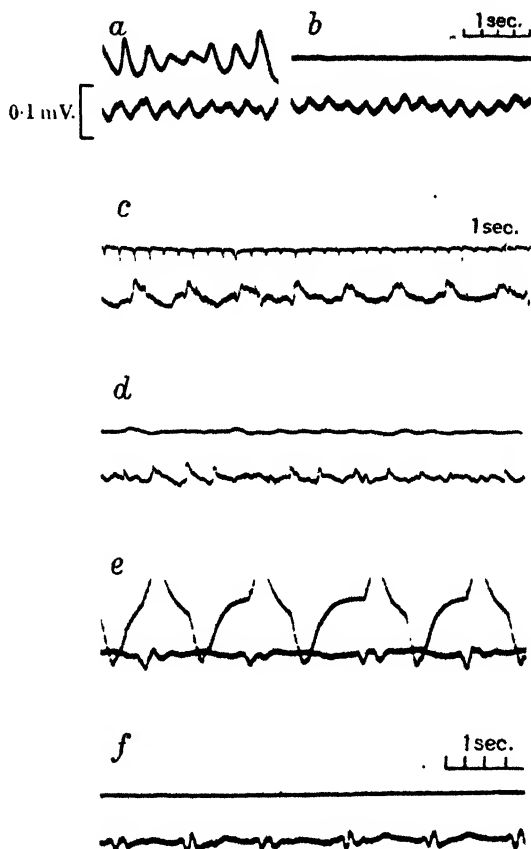
Fig. 6. Effect of inhibitory substances. Top line: photoelectric contraction-record, except in (*c*), where it indicates time. Bottom line: as before. (*a*) and (*b*) Mouse gut. Grid electrode, sewn on to the oral end, in paraffin; earth electrode not attached to the muscle, but immersed in Tyrode solution; top ⅔ of the preparation (2 cm. long) in the paraffin layer, bottom ⅓ in the Tyrode layer. (*a*) Initial activity. (*b*) Effect of adrenaline (10⁻⁵). Time in 1 sec. (*c*) and (*d*) Rabbit duodenum; usual fixation of electrodes (distance 1 mm.). (*c*) Initial activity (electrical change only; top line indicates time in 1 sec.). (*d*) Double recording; effect of 0·4 % CaCl₂; camera speed as in (*c*). (*e*) and (*f*) Another preparation. Rabbit duodenum; total length of the preparation 4 cm.; electrodes 4 mm. apart. Time in 1 sec. (*e*) Initial activity, showing contractions occurring in two halves; the electrical change precedes the second half-contraction. (*f*) Effect of 0·5 % CaCl₂. Inhibition is complete but the 'A' waves continue.

the contractions there is a great reduction in, and in places a disappearance of, the fast polyphasic response 'B', but 'A' continues unabated. In another

experiment (Fig. 6f), the inhibition produced by calcium was, for a time, complete, with a higher concentration than usual (0·5 % $CaCl_2$ = 25 × normal). Yet the 'A' waves (which showed reduplication in this experiment) continued; in fact, their frequency was slightly raised, from 11·5 to 15/min.

(b) *Adrenaline*. With adrenaline, although the mechanism of inhibition is different, the result is the same, both in rabbits (thus confirming an earlier observation of Berkson et al. 1932) and in mice (Fig. 6b).

(c) *Short periods of cooling*. On one occasion, the gut was left in the refrigerator for 3 hr. before the experiment. The preparation was then transferred to warm Tyrode solution in the bath and examined at intervals. After 25 min. muscular contractions were still in abeyance; nevertheless, slow rhythmic 'A' potentials of the usual type were observed at this stage, but fast impulses were absent. Half an hour later, i.e. 55 min. from the start of the experiment, vigorous pendulum movements had returned. Each contraction was now preceded by its 'A' wave, and fast impulses of type 'B' were also present during the contractions.

(d) *Chloroform*. When the Tyrode solution was saturated with chloroform, both the 'A' and 'B' types of activity were abolished.

The observations under headings (a), (b) and (c) all show that the slow potentials of type 'A' not only precede the pendulum movements but are also independent of the process of contraction, whereas the faster impulses 'B' appear to be more intimately associated with that process.

Effects of eserine

The effect of eserine on pendulum movements is difficult to analyse in fresh preparations because it also brings a reappearance of peristaltic reflexes. It was, therefore, convenient to examine the electrical changes produced by this drug in preparations cooled for 1 or 2 days, when the second action of eserine, which is undesirable in these experiments, is eliminated. Owing to the gradual decrease in the excursion of the pendulum movements produced by cooling, the records were taken with higher lever magnifications and a brighter light intensity of the beam falling on the photoelectric cell.

In the experiment of Fig. 7 a–c, the preparation was the same throughout, and the experiment was conducted in such a way as to avoid any alteration in the position of the electrodes from beginning to end. The initial activity of the gut was of the usual type (Fig. 7a), although the mechanical contractions tended to be slightly irregular. After the records were taken on the first day, the muscle was left in the bath and the whole bath was kept overnight in the refrigerator at 0–2° C. After cooling for 20 hr., the bath fluid was renewed and warmed to 37° C., and the preparation was re-examined. There was no appreciable change, at this stage, in the frequency of the rhythmic contractions, but they were smaller. At the same time, the electrical activity of the gut consisted
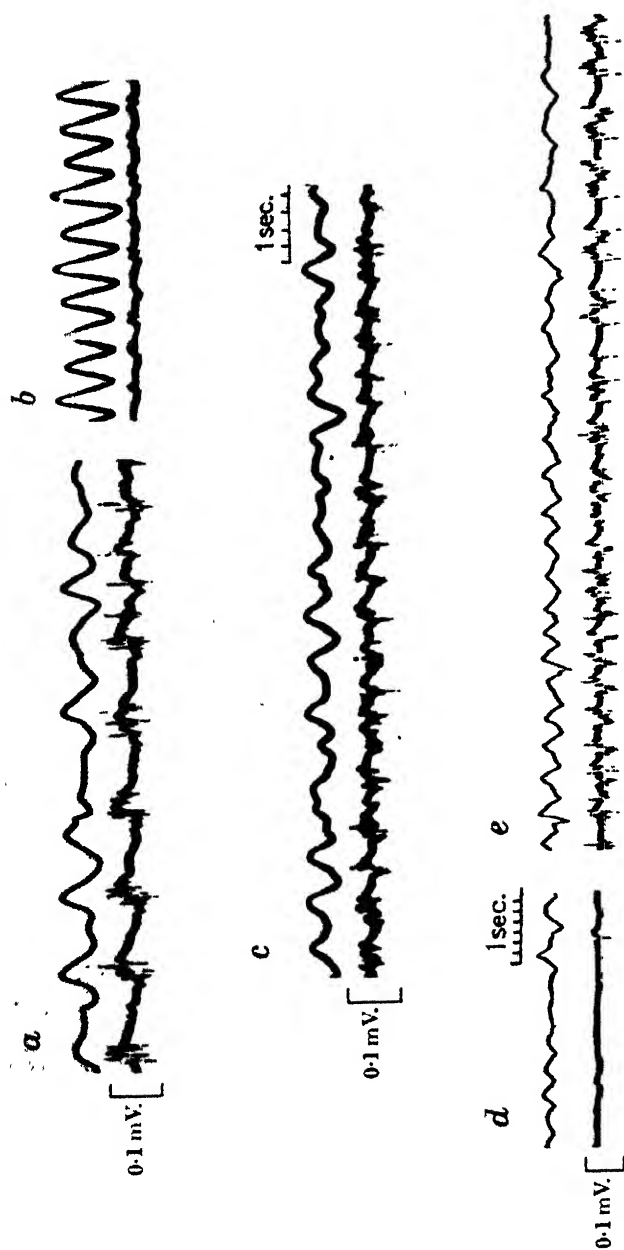
Fig. 7. Effect of cooling. (a), (b) and (c) From the same preparation (rabbit duodenum; electrodes 1·5–2 mm. apart). (a) Initial activity, before cooling. (b) Activity of the same preparation after cooling for 20 hr. at 0–2° C. Note disappearance of 'B' potentials. (c) Effect of eserine sulphate (4 × 10⁻⁷) showing return of spike potentials of type 'B'. The excursion of individual pendulum movements is smaller because the muscle is in a contracted state. (d) and (e) From another preparation (rabbit), cooled for 27 hr. at 0–2° C. Electrodes 1·5 mm. apart. (d) Initial activity of the cooled preparation. (e) Effect of eserine sulphate (8 × 10⁻⁷) introduced into the bath 6 mn. previously. Grouping of the 'B' potentials is seen in the latter half of the tracing.

mostly of 'A' waves (Fig. 7b); but in some experiments these were so small as to be barely detectable with the amplification available. In another experiment (Fig. 7d) there was after 27 hr. very little electrical activity. In both these experiments the addition of eserine (Fig. 7c, e) brought about a progressive increase in the electrical activity of the preparation, which was small at first, but very marked after a few minutes. The most noticeable change was the reappearance of spike potentials of type 'B', often clearly grouped, with each rhythmic contraction; this grouping is particularly well seen in the second half of Fig. 7e. A similar, though smaller, effect was recorded after 50 hr. cooling; at such a time, as Vogt (1943) has shown, the ganglion cells are functionless and it is unlikely that they are concerned in this effect.

Acetylcholine. The effect of acetylcholine was tried on preparations which were cooled previously for 2–4 days, so as to inactivate the ganglion cells, and thus eliminate the 'nicotine' action of acetylcholine. Although the muscle was shortened by acetylcholine and remained contracted, there was no detectable electrical activity, with the leads as close as 1 mm., even in preparations which later responded to eserine.

<div align="center">DISCUSSION</div>

In these experiments, two distinct forms of electrical activity have been found to accompany pendulum movements: an 'A' wave preceding each mechanical contraction and a polyphasic 'B' wave occurring during the contraction.

The polyphasic 'B' response appears to be most intimately associated with the contraction proper and can only be recorded when the intestine is actually contracting. Inhibition of pendulum movements by adrenaline or by excess of calcium ions, leads to a diminution and eventual disappearance of the 'B' electrical activity, and similar evidence of their close interdependence is provided by the increase in both mechanical and 'B' activity in the cooled gut after the administration of eserine.

It seems therefore difficult to escape the conclusion that the 'B' activity represents the action potentials of contracting muscle fibres. This view is supported by Fletcher's (1937) work on the action potentials of the anterior retractor of the byssus of *Mytilus edulis* and by Bozler's (1942) observations on the smooth muscle in the uterus and gut of various mammals.

The behaviour of the 'B' response when the distance between the recording electrodes is changed gives further support to this view. If Adrian's (1925) argument, based on experiments on the cat's tenuissimus, is applied, one would expect there to be no further broadening of the muscle action potential once the distance between the recording electrodes exceeded the length of a muscle fibre or active unit of the intestine. This, in fact, is what occurs; increased separation between the electrodes causes no obvious change in the shape of the 'B' potential, which would therefore appear to arise in units less than 1 mm. in length. In fact, the muscle fibres in the longitudinal layers of the rabbit's

intestine have been measured in conjuction with these experiments; in histological sections their average length is between 70 and 100μ.

The 'A' potentials present, on the other hand, an entirely different picture. Their onset precedes by a second or more the onset of contraction. They persist in intestine in which the pendulum movements have been inhibited by a variety of means. They show a clear dependence upon the distance between the recording electrodes, and it is difficult to avoid the conclusion that they are arising in some continuous conducting tissue with a fibre length much greater than that of the muscles concerned.

It is, of course, possible that the 'A' waves arise in muscles in which excitation is occurring without the appearance of internal work, such as occurs in the heart (Clark, 1938) or in the muscles of the crayfish (Marmont & Wiersma, 1938). Previous experiments (Ambache, 1946), however, have shown that a muscle in which movements have been stopped by calcium can still respond by contraction to acetylcholine and to electrical stimulation, and it appears unlikely that the 'A' wave could spread over tissue in this state of excitability without itself causing contraction.

If muscles are excluded as a source of the 'A' wave, the most likely tissue responsible for it in the gut would appear to be some nervous network. It seems improbable that Auerbach's plexus proper could be involved, since the wave persists after doses of nicotine sufficient to prevent transmission of excitation over synaptic junctions on ganglion cells.

The tissue which, in the author's opinion, is most likely to be involved is the nerve net which is present round Auerbach's plexus and in the interstices of the smooth muscle (Cajal, 1893, 1905). This structure seems to be identical with the 'nodal' tissue found by Keith (1915) in the rat's intestine. It is a truly syncytial structure consisting of the 'interstitial cells of Cajal' with their numerous anastomotic processes (Li, 1940).

Many of the phenomena observed in these experiments are compatible with the theory that such a nerve net acts as a pacemaker controlling pendulum movements and that its activity is manifested electrically in the 'A' wave. Such facts as the reversibility of conduction and the synchronization of the circular and longitudinal muscle layers of the gut (Bayliss & Starling, 1899; Trendelenburg's tracings, 1917) would be compatible with it. It would further explain the persistence of co-ordinated pendulum movements after nicotine, since this drug, in doses which paralyse synaptic ganglion cells, has no effect on other nervous tissues such as the dorsal root ganglia, the ganglion nodosum vagi and the bipolar nerve cells of the skate, all of which are non-synaptic (Langley & Dickinson, 1889; Langley, 1901).

Both Fischer (1944) and Rosenblueth & del Pozo (1942) have pointed out that smooth muscles form a heterogeneous class, and that comparisons between them may be misleading. This is very obvious when the electrical responses of

different smooth muscles are compared. For instance, a rhythmic component has been recorded electrically from the cat's nictitating membrane in response to single motor nerve volleys or to injections of adrenaline (Eccles & Magladery, 1937). These authors have suggested that 'adrenaline partly depolarizes the smooth muscle fibres, and hence renders them spontaneously rhythmic'. Yet, Rosenblueth & del Pozo (1942), who have criticized these deductions, failed to find any rhythmic component in the response of the pilomotors in the cat's tail, another muscle with an adrenergic innervation. These differences cannot be explained in our present state of knowledge. For instance, there is at present little information regarding the differences in the nerve-net innervation of the nictitating membrane and of the pilomotors, and any interpretation which does not take into account such possible differences between various smooth muscles is necessarily incomplete.

In comparing Eccles & Magladery's results with the above, it must be pointed out that their records were obtained with leads situated at either end of the nictitating membrane, i.e. many fibre lengths apart. In the present experiments it has been found that the muscle fibre potentials are considerably attenuated when the leads are so far apart. Also, with such an unrestricted lead it is impossible to obtain an action potential in skeletal muscle in response to injected acetylcholine (Brown, 1937), because the responses of the individual units are so out of phase as to cancel out; the failure to elicit, with acetylcholine, any electrical change from the gut in the present experiments could possibly be explained on similar lines. Yet, Eccles & Magladery obtained rhythmic responses to injected adrenaline, and they state that 'the various units are not responding independently. There must be some co-ordinating process by which they are kept in phase.' If it could be shown that a nerve net is also present in the nictitating membrane then Eccles & Magladery's findings would receive an explanation and their results could be brought into line with these.

SUMMARY

1. The electrical activity associated with pendulum movements has been recorded from isolated preparations of mammalian gut. An analysis of the records shows the existence of two distinct types of action potential.

2. There is, first, a slow diphasic wave, 'A', which *precedes* the onset of contraction by 0·5–1 sec. This electrical disturbance may start at either end of the preparation and is conducted for distances far greater than the length of one muscle fibre.

3. With electrodes close together there is also after the 'A' wave and *during* the contraction, a polyphasic response, 'B', consisting of faster diphasic spikes, the duration of which appears to be independent of the distance between the electrodes.

4. Both these responses persist after doses of nicotine which paralyse the ganglion cells.

5. When pendulum movements are inhibited by adrenaline or by an excess of calcium ions:

(*a*) The response 'B' disappears. It is thought that this polyphasic response consists of the action potential of asynchronous groups of muscle fibres.

(*b*) The 'A' waves continue. It is suggested that these represent the discharge of a pacemaker in the gut, and may arise in the nerve net which was described by Cajal (1905).

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THE DISTRIBUTION OF NUCLEOTIDE, PHOSPHOCREATINE AND GLYCOGEN IN THE HEART

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It has been shown (e.g. Davies & Francis, 1941a) that, contrary to common belief, the hearts of poikilothermal vertebrates (fish, Amphibia, reptiles) are devoid of those specialized muscular tissues—nodal and Purkinje tissues – that are responsible for the initiation and propagation of the stimulus for contraction in the hearts of mammals and birds, and that in each of these cold-blooded animals the musculature of the heart has the same histological characters throughout its several chambers. Nevertheless, when each chamber of the heart is separated from its neighbours it will continue to contract, but at a different rate from the others. This phenomenon is not confined to the poikilothermal vertebrates, and it is well known that the chambers of the mammalian heart beat at different intrinsic rhythms when the specialized tissues are inactivated. Since these different rhythmical rates cannot be explained on an anatomical basis, the possibility of chemical differences in the muscle fibres has been explored.

The role of adenosinetriphosphate (ATP) in the contraction of voluntary muscle has been established as a result of the work of Meyerhof, Parnas and their schools (for review, see Lipmann (1941)) and more recently of Szent-Györgyi (1942, 1943). The ATP content of voluntary muscle cannot be directly estimated, but an indication of its distribution can be obtained by estimating separately adenosine and pyrophosphate, since these substances do not exist in the free state but only in combination as adenine nucleotide (e.g. ATP) (Lohmann & Schuster, 1934; Umschweif & Gibayło, 1937). In cardiac muscle, ATP exists in combination with adenosinediphosphate (ADP), forming a dinucleotide (Embden, 1932; Beattie, Milroy & Strain, 1934; Ostern, 1934), but there is no evidence that this dinucleotide differs significantly from the mononucleotide of voluntary muscle in its physiological properties. The separate

estimation of adenosine and pyrophosphate should likewise give an indication of the distribution of the nucleotide complex in the heart. In voluntary muscle, ATP is present as the magnesium salt (Szent-Györgyi, 1943; Bielschowsky & Green, 1944); we have accordingly estimated the magnesium content of the cardiac chambers, since it is probable that some of the magnesium will be combined with the nucleotide, and the distribution of magnesium may therefore give a confirmatory indication of the distribution of the nucleotide.

As the difference in the intrinsic rhythms of the various cardiac chambers may be more related to the rephosphorylation of nucleotide during the recovery phase of the cardiac cycle than to its breakdown during contraction, the phosphocreatine and glycogen contents of the atria and ventricles have been estimated, since these substances are known to be involved in the rephosphorylation of nucleotide. That phosphocreatine may be important in the explanation of the differences in rhythm has already been suggested by Clark, Eggleton, Eggleton, Gaddie & Stewart (1938).

METHODS

Hearts from the frog, pigeon, rabbit and ox were used. Except where specially mentioned, the rabbits and pigeons were killed by a blow on the head and the frogs were pithed. The results have been expressed in mg./g. wet weight. Owing to the small amounts of tissues available, it was not possible to determine the dry weight in each analysis. The water content of the atria and ventricles has, however, been determined in a separate series of animals for each species, and the differences between the two chambers were found to be negligible in every case.

Nucleotide estimations. In the frog, the heart was perfused (in situ) with isotonic NaCl solution and the ventricle and atria removed in one piece and dropped on to filter paper; the atria were then cut apart from the ventricle and each of these two segments was weighed on a torsion balance and then ground with quartz and 5 % trichloroacetic acid (1·0 ml. 0·1 g. muscle), the two mortars being kept in ice. The atria and ventricles were thus accumulated in the mortars until sufficient material had been obtained for an estimation. For an individual frog this process was completed in 2–3 min.

In the rabbit and pigeon, the heart was removed and perfused through the coronary arteries with isotonic NaCl solution to wash out the blood; the two atria and an approximately equal mass of the ventricles were then cut away, blotted, weighed on chilled watch-glasses and ground up with trichloroacetic acid, the whole process taking 5–6 min.

The ox hearts were handed to us within 4 min. of the animals having been shot. They were not perfused, but samples of the right atrium, right and left ventricles, atrio-ventricular bundle and the sinu-atrial region of the right atrium were excised, washed rapidly in isotonic NaCl solution, blotted, weighed and ground in trichloroacetic acid. These operations were carried out as quickly as possible. The dissection of the atrio-ventricular bundle was the slowest operation, but was accomplished within 10 min. of the death of the animal.

The trichloroacetic acid extracts were filtered and stored in the refrigerator overnight to permit the hydrolysis of any phosphocreatine present (Lohmann, 1928), which otherwise might have interfered with the phosphate determinations. The adenosine equivalent of neutralized portions of the filtrates was determined on the guinea-pig atrium preparation of Drury, Lutwak-Mann & Solandt (1938) as modified by Stoner & Green (1944). This method estimates adenosine and its phosphorylated derivatives, except yeast adenylic acid, and is accurate to ± 10 %. Cytidylic

acid was not present in sufficient quantity in our extracts to interfere with the estimation (cf. Drury *et al.* 1938). The pyrophosphate content of the filtrates was determined by the difference between the inorganic phosphate present before and after 7 min. acid hydrolysis, i.e. the phosphate liberated by the 7 min. hydrolysis. Although there is no unanimity of opinion that the phosphate thus estimated is derived solely from ATP, it should be observed that we are at present mainly concerned with relative rather than absolute values, and the results estimated in the different samples by the same method are probably comparable. The phosphate estimations were performed by Briggs' method (Peters & Van Slyke, 1932). The total magnesium content of the filtrates was determined by Lang's method (1932).

Phosphocreatine and creatine estimations. For these determinations the hearts were removed as rapidly as possible, and the atria and ventricles were then separated, blotted and plunged into liquid air. For all these operations the time taken did not exceed 1 min. for any animal. Since Burns & Cruickshank (1937) noted that the rate of phosphocreatine breakdown in the hearts of the cat and dog during anoxaemia was more rapid in the ventricles than the atria, the first portions of tissue to be placed in liquid air in our experiments were taken alternately from the atria and the ventricles. In some of the rabbits, the hearts were removed under artificial respiration, the animals being anaesthetized with ether. When frozen, the tissues were weighed and the phosphocreatine P content determined by the method of Eggleton & Eggleton (1929). Portions of the trichloroacetic acid extracts used in this method were allowed to stand at room temperature for 1 hr. to permit hydrolysis of the phosphocreatine, and the total creatine content was then determined by the method of Eggleton, Elsden & Gough (1943). Portions of these extracts were also used in studying the nucleotide distribution between the right and left ventricles of the rabbit, the nucleotide being estimated as above.

Glycogen estimations. Glycogen was determined by the method of Good, Kramer & Somogyi (1933). The hearts were rapidly removed and the atria and an approximately similar mass of the ventricles were dissected free, blotted, weighed on a torsion balance and dropped into tubes containing 2·0 ml. hot 30 % KOH. The time taken for these operations did not exceed 90 sec. for any animal. In experiments to determine the rate of breakdown of glycogen in the atria and ventricles, the heart was rapidly removed and portions of atria and ventricles added to the KOH at intervals after removal. In some cases the separation of the glycogen into its 'bound' and 'free' forms was performed according to the method of Wajzer (1939). When this was done on the frog's heart, the atria and ventricles were stored in liquid air until sufficient tissue had been accumulated for an estimation.

<div align="center">RESULTS</div>

Nucleotide distribution

The results of the estimations of the different fractions of the nucleotide complex in the atria and ventricles of the frog, pigeon, rabbit and ox are shown in Table 1.

In all the hearts the adenosine fraction was present in greater amount in the ventricles than in the atria, i.e. it showed a positive correlation with the work done by the heart muscle of these chambers. It is rather striking that, as regards the two widely dissimilar mammals (rabbit and ox), the contents of adenosine were closely similar in the corresponding parts of the heart, and that in all the homoiothermal vertebrates examined (rabbit, ox and pigeon) the ratio between the adenosine content of the ventricles and of the atria showed no great disparity. In the poikilothermal heart (frog), however, whereas the adenosine content of the ventricular muscle was approximately the same as in the rabbit and ox, that of the atria was considerably less than

that in the mammal; so that the ventriculo-atrial ratio (V/A) for adenosine was much higher in the frog than in the mammal. Whereas the V/A ratio for adenosine in the pigeon was about the same as that in the rabbit and ox, the adenosine contents of both the atria and the ventricles of the pigeon were much greater than those of the corresponding chambers of the mammal.

TABLE 1. The nucleotide distribution in the atria and ventricles of the frog, pigeon, rabbit and ox

Species	No. of animals	Tissue	Adenosine content mg./g. wet wt.	Pyro- phosphate content expressed as mg. P/g. wet wt.	Magnesium content mg./g. wet wt.	V/A ratio		
						A	7′ P	Mg
Frog	28	Atria	0·25	0·55	1·28			
		Vent.	0·86	0·29	0·35	3·45	0·53	0·37
	27	A.	0·48	0·15	—			
		V.	1·25	0·15	—	2·60	1·00	—
	18	A.	0·61	0·22	2·10			
		V.	1·86	0·11	1·14	3·05	0·50	0·54
Pigeon	3	A.	—	0·092	0·28			
		V.	—	0·102	0·39	—	1·13	1·40
	3	A.	2·07	0·062	0·39			
		V.	2·95	0·118	0·35	1·43	1·91	0·90
	2	A.	—	0·12	0·43			
		V.	—	0·13	0·54	—	1·08	1·26
	2	A.	1·80	0·123	0·75			
		V.	3·00	0·178	0·96	1·67	1·45	1·28
	2	A.	1·13	0·15	0·89			
		V.	1·43	0·18	0·79	1·27	1·20	0·89
Rabbit	2	A.	—	0·07	—			
		V.	—	0·12	—	—	1·75	—
	2	A.	0·67	0·15	—			
		V.	1·00	0·20	—	1·5	1 35	—
	3	A.	—	0·16	—			
		V.	—	0·29	—	—	1·80	—
	1	A.	0·78	—	0·54			
		V.	1·10	—	0·67	1·42	—	1·24
	1	A.	0·74	—	0·50			
		V.	1·50	—	0·82	2·03	—	1·64
	1	A.	—	—	0·66			
		V.	—	—	0·79	—	—	1·20
Ox	1	R. atrium	0·86	0·08	0·13			
		R. vent.	1·30	0·11	0·22	1·52	1·34	1·70
	1	R. atrium	0·80	0·09	0·25			
		R. vent.	1·40	0·11	0 31	1·75	1·23	1·24

The pyrophosphate contents of the atria and ventricles of the rabbit, ox and pigeon were somewhat similar, the V/A ratio in each case being greater than unity; but in the frog, while the ventricular pyrophosphate was similar to that in the mammals and bird, the atrial pyrophosphate was much greater than the ventricular, so that the V/A ratio was less than unity.

The amount of magnesium present in the atria and ventricles was closely similar in the rabbit and pigeon; in the ox, it was very low in both chambers, but in all three animals the V/A ratio was greater than unity. On the other hand, in the frog, as in the case of the pyrophosphate, whereas the magnesium content of the ventricle was comparable with that of the rabbit and pigeon, the atrial content was much higher than the ventricular, making the V/A ratio less than unity.

TABLE 2. The nucleotide distribution in the ventricles of the rabbit and ox

Species	No. of animals	Ventricle	Adenosine content mg./g. wet wt.	Pyrophosphate content expressed as mg. P/g. wet wt.	Magnesium content mg./g. wet wt.
Rabbit	2	Right	1·75	0·30	—
		Left	2·03	0·14	—
	2	R.	1·70	0·19	—
		L.	2·14	0·15	—
	2	R.	2·18	0·24	—
		L.	1·95	0·27	—
	2	R.	1·24	0·25	—
		L.	1·40	0·20	—
	2	R.	3·30	0·15	—
		L.	2·65	0·19	—
	2	R.	0·84	0·19	—
		L.	2·10	0·19	—
	Average	R.	1·84	0·22	—
		L.	2·05	0·19	—
Ox	1	R.	1·30	0·11	0·22
		L.	1·50	0·11	0·24
	1	R.	1·40	0·11	0·31
		L.	1·50	0·10	0·26
	Average	R.	1·35	0·11	0·27
		L.	1·50	0·105	0·25

If, as suggested above, the nucleotide content of a cardiac chamber is correlated with the amount of work performed, it becomes of interest to compare the two ventricles (Table 2). In the rabbit and ox the adenosine content of the left ventricle was greater than that of the right. The greater difference in the rabbit may arise because the method of extraction in these experiments was better capable of revealing a small difference in the rabbit than in the ox (see under Methods). There was no significant difference between the pyrophosphate and magnesium contents of the right and left ventricles. Of the three tests, however, the adenosine equivalent is by far the most sensitive and thus affords the most accurate indication of the nucleotide distribution. Nevertheless, the difference between the adenosine content of the right and left ventricles is too near the limits of experimental error for undue significance to be attached to it.

TABLE 3. The nucleotide distribution in the specialized tissue of the ox heart

Tissue	No. of animals	Adenosine content mg./g. wet wt.	Pyrophosphate content expressed as mg. P/g. wet wt.	Magnesium content mg./g. wet wt.
Sinu-atrial node	3	0·63	0·05	0·13
	2	0·61	0·18	0·18
	2	0·53	0·17	0·16
Atrio-ventricular bundle	3	0·77	0·12	0·36
	2	0·64	0·21	0·397
	2	0·56	0·20	0·33

The nucleotide distribution in the specialized muscle tissue of the ox heart is shown in Table 3. Both the sinu-atrial (s.a.) node and the atrio-ventricular (a.v.) bundle contained less adenosine than the ordinary cardiac muscle; the pyrophosphate and magnesium contents of the s.a. node did not differ markedly from the atrial muscle, while those of the a.v. bundle resembled the ventricular muscle.

Total creatine and phosphocreatine distribution

The absolute values obtained for the phosphocreatine P (Table 4) were similar to those obtained by other workers (Clark *et al.* 1938, in the frog; Chang, 1937, and Burns & Cruickshank, 1937, in the rabbit). We found in the rabbit, as Pollack, Flock & Bollman (1934) had observed in the dog, that very little phosphocreatine was recovered unless the heart was removed under artificial respiration. Although the total creatine content of the ventricles was greater than that of the atria in each of the three animals studied, the phosphocreatine P showed the reverse relationship. This difference is further brought out when the percentage of the total creatine existing in the phosphorylated form is calculated. Although the figures for this percentage are variable they show that there is always more phosphorylated creatine available in the atria than in the ventricles. The figures of Chang (1937) and of Burns & Cruickshank (1937) also show that there is more phosphocreatine P in the atria than in the ventricles of the mammal, but these authors did not comment on the difference. We have found that in most cases (four out of six) the right ventricle of the rabbit contained slightly more phosphocreatine P than the left, but the difference may not be significant. since its magnitude falls at the margin of the experimental error as determined by analysis of duplicate samples from the same ventricle.

Glycogen distribution

The total glycogen contents of the atria and ventricles of the pigeon and rabbit are shown in Table 5. For completeness, the previous chemical results obtained by Davies & Francis (1941*b*) in the frog have been included; the

TABLE 4. Total creatine and phosphocreatine P contents of atria and ventricles of frog, pigeon and rabbit

Species	No. of animals	Tissue	Creatine content mg./g. wet wt.	V/A ratio	Phospho-creatine P mg./g. wet wt.	V/A ratio	% of total creatine in phos-phorylated form
Frog	30	Atria	0·46		0·049		50·0
		Vent.	1·24 .	2·7	0·021	0·43	7·2
Pigeon	2	A.	0·91		0·010		4·6
		V.	2·17	2·4	0·010	1·00	1·9
	2	A.	0·55		0·040		31·0
		V.	3·03	5·56	0·020	0·50	2·8
	3	A.	1·0		0·020		8·5
		V.	1·84	1·84	0·010	0·50	2·3
	2	A.	—		0·007		—
		V.	—	—	0·006	0·86	—
	2	A.	—		0·020		—
		V.	—	—	0·019	0·95	—
	2	A.	—		0·028		—
		V.	—	—	0·012	0·43	—
	2	A.	—		0·013		—
		V.	—	—	0·012	0·92	—
	2	A.	—		0·016		—
		V.	—	—	0·010	0·62	—
	2	A.	—		0·020		—
		V.	—	—	0·015	0·75	—
Rabbit	2	A.	0·97		Trace		—
		V.	1·12	1·16	Trace	—	—
	2	A.	0·85		Trace		—
		V.	1·28	1·50	Trace	—	—
	2	A.	0·76		Trace		—
		V.	1·28	1·68	Trace	—	—
	2*	A.	0·91		0·060		28·0
		V.	1·37	1·5	0·060	1·00	18·5
	1*	A.	0·85		0·030		15·0
		V.	1·28	1·5	0·040	1·33	13·2
	2*	A.	1·44		0·030		8·8
		V.	2·25	1·56	0·020	0·66	3·75
	2*	A.	0·99		0·050		21·5
		V.	1·92	1·94	0·030	0·6	6·6
	2*	A.	—		0·017		—
		V.	—	—	0·019	1·12	—
	2*	A.	—		0·033		—
		V.	—	—	0·038	1·15	—
	2*	A.	—		0·025		—
		V.	—	—	0·019	0·76	—
	2*	A.	—		0·019		—
		V.	—	—	0·019	1·00	—
	2*	A.	—		0·021		—
		V.	—	—	0·021	1·00	—
	2*	A.	—		0·019		—
		V.	—	—	0·009	0·5	—

* Hearts removed under artificial respiration.

TABLE 5. Total glycogen content of atria and ventricles of frog, pigeon and rabbit

Species	No. of animals	Tissue	Glycogen content mg./g. wet wt.	V/A ratio
Frog (Davies & Francis, 1941 *b*)	6	Atria Vent.	7·8 18·5	2·37
	6	A. V.	6·2 15·2	2·45
Pigeon	2	A. V.	2·06 2·48	1·20
	2	A. V.	0·93 1·32	1·41
	2	A. V.	2·51 2·25	0·89
	2	A. V.	1·02 4·30	4·20
	1	A. V.	2·88 5·62	1·95
	1	A. V.	1·28 3·78	2·95
	1	A. V.	4·31 6·75	1·56
	1	A. V.	2·07 3·58	1·73
	1	A. V.	3·22 6·78	2·10
Rabbit	1	A. V.	3·90 3·25	0·83
	1	A. V.	4·88 3·49	0·73
	1	A. V.	6·17 5·85	0·95
	1	A. V.	6·47 4·07	0·63
	1	A. V.	6·13 6·48	1·07
	1	A. V.	6·02 4·97	0·82
	1	A. V.	5·11 4·20	0·82
	1	A. V.	8·55 5·98	0·70
	1	A. V.	6·85 5·25	0·77
	1	A. V.	4·62 3·52	0·76
	1	A. V.	9·04 7·84	0·87

histochemical methods used by them are not suitable for investigating the distribution of glycogen in the pigeon and rabbit. Although in the frog the total glycogen content of a cardiac chamber appears to be correlated with the amount of work done, in that there is more in the ventricle than in the atria, this difference is less noticeable in the pigeon, while in the rabbit the total glycogen content of the atria is slightly more than that of the ventricles. In the rabbit the right ventricle contains slightly more glycogen than the left ventricle (six cases); on the average the right ventricle contained 4·00 mg./g. wet weight as compared with 3·68 mg./g. for the left ventricle. This difference is of the same order as that found between the ventricles of the dog by Cruickshank & Shrivastava (1930), but its significance is doubtful since it falls just within the margin of experimental error as determined by analysis of duplicate samples from the same ventricle. Berblinger (1912) states that the atria of man contain more glycogen than the ventricles.

TABLE 6. 'Free' and 'bound' glycogen contents of the atria and ventricles of the pigeon, rabbit and frog

Species	No. of animals	Tissue	Glycogen content mg./g. wet wt.		V/A ratio		% of total glycogen in 'free' form
			'Free'	'Bound'	'Free'	'Bound'	
Pigeon	1	Atria	4·32	2·03			68
		Vent.	0·71	2·04	0·16	1·0	26
	1	A.	4·08	2·61			61
		V.	1·51	3·42	0·37	1·31	31
	1	A.	2·60	1·50			63
		V.	1·83	1·70	0·70	1·13	52
	1	A.	2·06	0·89			70
		V.	0·50	0·82	0·24	0·93	38
Rabbit	1	A.	1·45	1·06			58
		V.	0·50	0·50	0·34	0·47	50
	1	A.	4·21	2·80			60
		V.	2·94	1·87	0·70	0·67	61
	1	A.	5·30	2·49			68
		V.	1·63	1·62	0·31	0·65	50
	1	A.	3·80	2·74			58
		V.	2·08	1·71	0·55	0·63	55
Frog	20	A.	5·48	2·44			69
		V.	7·40	4·80	1·36	1·97	61

The rate of glycogenolysis in the homoiothermal animal is very much faster than in the frog (Clark et al. 1938; Evans, 1934), and the smaller amounts of glycogen in the hearts of the pigeon and rabbit may be a reflexion of this. The work of Berblinger (1912) suggests that the rate of glycogenolysis may be faster in the ventricles than in the atria, but we found no difference in the rate of disappearance of glycogen in the atria and ventricles of the pigeon and rabbit after their removal from the body. Consequently the apparent non-conformity of the V/A ratio for total glycogen in the rabbit cannot be explained

by a faster rate of breakdown in the ventricles. The rate of breakdown of cardiac glycogen in these animals does not appear to be as rapid as that reported for the rat by Evans (1934).

Since the glycogen in the heart exists only partly in the 'free' form, immediately available for participation in chemical reactions, the rest being stored, the total glycogen content of a chamber may not be a reliable indication of the glycogen immediately available for use in muscular contraction. An attempt was therefore made to differentiate between these two forms (Table 6). In the pigeon and rabbit, although the amount of glycogen stored in the atria and ventricles was about the same, the amount of 'free' glycogen was greater in the atria. In the frog there was more 'free' glycogen in the ventricle than in the atria, but even in this animal the *percentage* of the total glycogen present in the 'free' form was greater in the atria.

<div style="text-align:center">DISCUSSION</div>

The available evidence suggests that muscular contraction is associated with the dephosphorylation of ATP, rephosphorylation occurring during the breakdown of phosphocreatine and glycogen. Although, in cardiac muscle, ATP may exist in combination with ADP, there is no reason to suppose that it behaves differently from the form in voluntary muscle.

As an indication of the nucleotide content of cardiac muscle we have separately estimated adenosine, pyrophosphate and magnesium. These substances were not present in the same ratio in all the hearts examined: for instance, although pyrophosphate and magnesium were present to about the same extent in the corresponding chambers of the pigeon and rabbit hearts, there was more than twice as much adenosine in the bird's heart. Similarly when the rabbit and frog were compared the proportions of all three substances were about the same in the ventricles but they differed in the atria. These results suggest that the nucleotide complex is not identical in all the species examined.

Despite this, in all the hearts examined, the adenosine compound was clearly present in greater amount in the ventricles than in the atria and its distribution may therefore be correlated with the work done by the musculature of these chambers. The close correspondence between the nucleotide contents of the chambers of the ox and rabbit hearts suggests that the amount of work normally done per gram per beat may be approximately the same in the corresponding chambers, and it is of interest to find (Clark, 1927) that such is the case for the left ventricular muscle of a variety of mammals. Comparable figures are not available for the work done by the frog's heart, where the ventricular nucleotide content resembles that of the mammal, nor for the bird where the ventricular content is much greater.

In the specialized conducting tissues of the mammalian heart (ox) there would seem to be less nucleotide than in the myocardium. Whilst the results obtained probably reflect the true conditions in the dissected A.V. bundle the significance of the results for the S.A. node is difficult to evaluate, since the specimens were inevitably contaminated with fibrous tissue and ordinary atrial muscle.

The results obtained for the total creatine and phosphocreatine estimations are of interest since they show that, although the total creatine content of a chamber varies with the amount of work it does, i.e. more in the ventricles than atria, the phosphorylated creatine content varies inversely with the work done by the chamber but directly with its intrinsic rhythmic rate, i.e. more in the atria than in the ventricles. The phosphocreatine contents found here may be lower than actually exist in vivo since this compound breaks down under anoxaemic conditions. The factor of anoxaemia is especially important since Burns & Cruickshank (1937) showed that the ventricular content of phosphocreatine was more affected by asphyxia than the atrial content. In our experiments the influence of anoxaemia was excluded as far as possible and we obtained the same difference between atrial and ventricular contents as did Burns & Cruickshank in tissue which they considered normal. Consequently the difference reported here is believed to be a real one.

It was previously found in the frog (Davies & Francis, 1941b) that the total glycogen content of a cardiac chamber varied inversely with its intrinsic rhythmic rate and directly with the work done. Whilst a similar relationship was found in the pigeon's heart, in the rabbit the atria contained more total glycogen than the ventricles. However, when the 'free' portion of the total glycogen was measured, these species' differences were seen to be due largely to the stored (protein-bound) glycogen. In the pigeon and rabbit the atria contained more 'free' glycogen than the ventricles, and even in the frog more of the atrial than ventricular glycogen occurred in the 'free' form. The distribution of the 'free', reactive, cardiac glycogen appears therefore to be related to the intrinsic rhythm of the chamber.

The differences between the atrial and ventricular contents of the substances examined cannot be explained by differences in the water contents of the atria and ventricles for these were found to be negligible. Furthermore, the differences between the atrial and ventricular concentrations of the compounds were not in the same direction in all cases. This latter observation also refutes any contention that the differences may be due to the amounts of connective tissue present in the different chambers (see also Davies & Francis, 1941b).

The principal facts emerging from the present study are that the ventricles have a greater store of potential energy than the atria, containing greater amounts of nucleotide, whilst the distribution of the substances from which the dephosphorylated nucleotide regains its phosphate groups can be more

closely related to the intrinsic rate of the chamber, in that they are present in greater amount in the atria than in the ventricles. It may be deduced from this that the larger amounts of phosphate donators in the atria might account for their higher rate. For, since the speed of nucleotide breakdown is likely to be the same in both atria and ventricles, the frequency at which this breakdown can recur is probably dependent upon, and will be limited by, the rate at which nucleotide can be resynthesized, and this might occur more rapidly in the atria than in the ventricles since in the former lie greater stores of phosphate donators. Certainly the absolute refractory period of the atria is shorter than that of the ventricles.

To draw these conclusions without reserve would be to overlook the shortcomings of the experimental methods applied. The results described here show the amounts of these substances present at any one time in the different parts of the heart, they do not give any information of the rate at which these compounds are reacting, without which the full significance of the results cannot be assessed. Again, the amount of phosphorylated compounds and glycogen in a cardiac chamber will vary with the phase of the cardiac cycle. The pyrophosphate, phosphocreatine P and glycogen determinations will be most affected by this, and under our experimental conditions this difficulty could not be circumvented, nevertheless, the relative constancy of the V/A ratios should be noted. Although the thicker mass of ventricular muscle will take slightly longer than the atrial muscle to cool completely to the temperature of liquid air, we do not think that this explains our finding less phosphocreatine P in the ventricles, because the temperature of liquid air is so much below that required to stop the breakdown of phosphocreatine that the time difference between the cessation of this reaction in the atria and ventricles will be negligible. A further difficulty is that the atria and ventricles do not normally contract at their intrinsic rates since they are driven considerably faster by the sinus (frog) or S.A. node (mammal and bird). There is therefore for each chamber an interval between the end of its absolute refractory period and the onset of the next *spontaneous* contraction during which contraction is possible on the application of an external stimulus. What happens during this interval — whether nucleotide is slowly building up to a maximum necessary to induce spontaneous contraction, or whether some other substance acting as an internal stimulus is being synthesized—remains to be determined. Nevertheless, by showing the presence of chemical differences where there is histological uniformity, we believe that these results do form an approach to the problem of the different intrinsic rhythms of the cardiac chambers.

SUMMARY

1. The nucleotide, phosphocreatine and glycogen contents of the atria and ventricles of the frog, pigeon, rabbit and ox have been examined.

2. In all the animals studied, the ventricles were found to contain more nucleotide than the atria per g. wet weight. The nucleotide content of the specialized initiating and conducting tissue of the ox heart was less than that of cardiac muscle.

3. The total creatine content of the ventricles was greater than that of the atria, but the percentage of this substance existing in the phosphorylated form was greater in the atria.

4. The total glycogen content of the ventricles was greater than that of the atria in the frog and pigeon, but not in the rabbit where more was present in the atria. In all these animals the percentage of total glycogen in the 'free' form was greater in the atria than the ventricles.

5. These differences could not be attributed to differences in the water or fibrous tissue contents of the chambers.

6. The relationship of these findings to the problem of the different intrinsic rhythms of the cardiac chambers has been discussed.

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STUDIES ON THE EFFECT OF *l*-TYROSINE
ON THE WHITE RAT

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For some years amino-acids have been employed in therapy either alone or in various combinations. Because of this, the importance of a series of investigations by various authors, which demonstrate that amino-acids can injure laboratory animals, has increased. Among such amino-acids, tyrosine takes a special position. It probably has a close chemical and physiological relationship with thyroxine, adrenaline and melanin. In addition, the metabolism of tyrosine is involved in such pathological states as alkaptonuria, tyrosinosis (Medes, 1932) and phenylpyruvic imbecility (Fölling, 1934). Abderhalden & Kankeleit (1917) described the production of renal infarction in rabbits by tyrosine feeding. Kotake, Matsuoka & Okagawa (1922) observed that small quantities of tyrosine can produce acute nephritis in rabbits and kill them. Newburgh & Marsh (1925) also demonstrated the nephrotoxic action of tyrosine on dogs and rabbits. Shambough & Curtis (1927) and Shambough (1927, 1929) found necrotic changes in the liver of rabbits and white rats treated with tyrosine. Oehme (1939) showed that tyrosine can cause the death of guinea-pigs. In poultry, tyrosine retards growth (Hill & Slinger, 1945). Sullivan, Hess & Sebrell (1932) observed that feeding with tyrosine ($2\frac{1}{2}$–20% of a basal diet low in protein) produced characteristic symptoms in white rats: damage to the eyes, redness and swelling of the extremities, loss of weight and shortening of the length of life. There were necrotic changes in the kidneys (Lillie, 1932). Martin (1943), Martin & Hueper (1943) and Hueper & Martin (1943) confirmed these results in experiments with black rats receiving a basal diet richer in protein. They further proved that the first stages of tyrosine poisoning are characterized by an elevation of the blood pressure, the later stages by a hyperglycaemia. The chief histopathological changes were necrotizing lesions of the pancreas and kidneys; the arterioles of the brain, heart, lungs and kidneys exhibited swelling and hyalinization of the tunica media. From this the authors suggested that tyrosine produces a hypertensive state, which could partly explain the appearance of these symptoms.

The investigations described below, summarized partly in a preliminary report (Schweizer & Zeller, 1946) have been carried out to illustrate the effect of tyrosine on the white rat and to elucidate the possible role of the thyroid gland in the mechanism of action of tyrosine.

METHODS

White rats were used. They were housed in individual glass cases and weighed three times a week. The number of the animals used in each experimental group, their age and weight at the beginning of the experiments are mentioned with the results.

The basal diet consisted of potatoes, fat and yeast and had the following percentage composition: carbohydrates 80, proteins 12, fat 8. It contained thiamin 60 μg., riboflavin 15 μg., nicotinic acid 75 μg., pyridoxine 5 μg., pantothenic acid 20 μg./100 g. Daily supplements of cod-liver oil (0·005 c.c., 1500 i.u./c.c.) and choline (0·003 g.) were given in addition to fresh water. The diet was supplied ad libitum each day and records were kept of the food consumed. The tyrosine content of the proteins of the basal diet amounted to 100 mg. %. Young rats grow well on this diet without any symptoms of deficiency. Pure *l*-tyrosine, isolated from protein hydrolysates, was mixed in different quantities with the diet.

Oxygen consumption was expressed as c.c./sq.m. body surface/24 hr. Surface area, S, was calculated from the Meeh-Rubner formula, $S = k \sqrt[3]{g^2}$, where $k = 9·1$ (von Muralt, 1944) and $g = $ wt. in g. Each determination was preceded by a fasting period of 15–20 hr. The examinations were carried out on the 1st, 7th, 14th, 21st and 28th days.

The bulbi oculorum, thyroid glands and toes of a number of animals were examined after tyrosine feeding (paraffin/celloidin; hematoxylin-eosin). The investigations were carried out after spontaneous death or after an experimental period of 40 days.

The degree of activity of the thyroid glands was estimated according to the system of Kampelmann (1936). The criteria (size of follicles, form of cells, position of the nuclei, quantity and colour of the colloid) were compared with those obtained from the examination of the thyroid glands of ten healthy rats of the same age fed on the same basal ration.

RESULTS

Effects on young white rats

195 rats (111 ♂♂, 84 ♀♀), average initial weight 67 (50–100) g., average age 51 (30–70) days. Tyrosine was mixed with the basal diet in the following amounts: 0·5 % (22 rats); 1·0 % (47 rats); 1·5 % (32 rats); 2·0 % (44 rats); control rats: 50.

The quantity of tyrosine eaten daily by the animals of each experimental group was obtained by recording the food consumption. The quantities (per 100 g. body wt.) remained nearly constant throughout the experiment and were 0·14–0·15, 0·28–0·31, 0·43–0·48 and 0·56–0·62 g. respectively.

The smallest amount of tyrosine, applied orally over several days, which is capable of injuring the rats has been proved to be between 0·2 and 0·3 g./100 g. body wt. per day.

Effect on growth and length of life

Tyrosine diminishes the rate of increase in weight of young rats (Text-fig. 1). This diminution is not caused by lessening appetite. Tyrosine shortens the life of young rats. Within 40 days, 17 % of the rats which received an addition of 1 % tyrosine to the basal diet died after an average life of 36 days. With an addition of 1·5 %, the corresponding values were 69 % and 28 days, with 2·0 % it was 91 % and 20 days. The death-rate of the animals in each group is indicated in Text-fig. 2.

Development of a characteristic syndrome

Keratitis and conjunctivitis. The first signs of damage of the eyes are small multiple or diffuse erosions of the epithelium of the cornea; the stroma appears oedematous. Then follows a progressive cloudiness of the cornea caused by swelling and cellular infiltration. Vascularization of the cornea begins 2–5 days after the appearance of the first signs.. As a rule, the cloudiness is reduced within 14–19 days, and the blood in the newly formed vessels disappears, although the tyrosine feeding continues. A diffuse transparent cloudiness (macula) remains. The process has a certain similarity to the interstitial keratitis, associated with congenital syphilis (Pl. 1, fig. 1). The recovery may last, but in some cases, a second period of cloudiness occurs after some days. This also
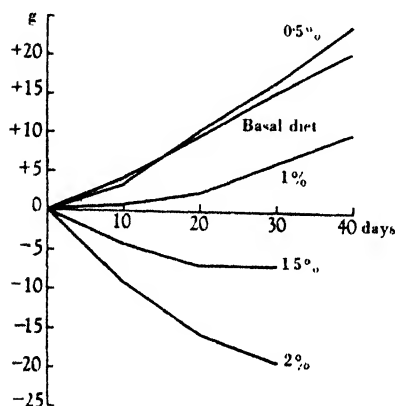
Text-fig. 1. Change in weight of young white rats receiving tyrosine.
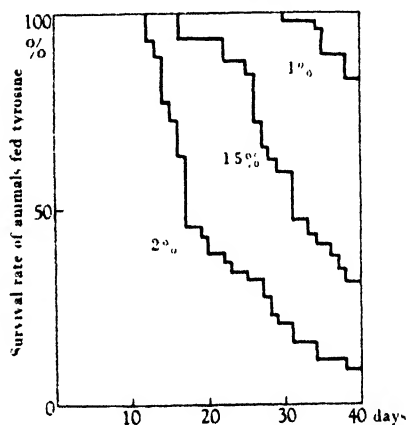
Text-fig. 2. Shortening of the length of life of young white rats fed tyrosine.

may clear up, and a third period of keratitis may follow. The keratitis is usually bilateral, though one eye may be affected a few days before the other. It is always bilateral when 2·0% tyrosine is used.

Histological examination shows oedema and cellular infiltration and, in advanced cases, a close capillary network. The changes are confined to the epithelium.

With an addition of 1% tyrosine to the diet 78% of the rats show keratitis after an average of 19 days. With 1·5% tyrosine the corresponding figures are 100% and 13 days, with 2·0%, 100% and 9 days. Conjunctivitis is usually found in these cases and is invariable when 2·0% tyrosine is added. The disappearance of the keratitis is generally accompanied by an improvement of the conjunctivitis.

Alopecia. Tyrosine causes loss of hair in large symmetrical patches. The skin is smooth and without any signs of inflammation. The size of the patches generally increases in proportion to the amount of tyrosine used.

Within 40 days, 49% of the rats receiving 1% tyrosine showed loss of hair after an average of 21 days. The corresponding values for the groups with 1·5 and 2·0% tyrosine were 69% and 15 days and 61% and 12 days.

Cheilitis. Tyrosine produces inflammatory processes on the lips. Smaller amounts cause erythema and swelling; with greater quantities, exudative inflammation appears (Pl. 1, fig. 2).

With 1·0% tyrosine in the diet, these effects are observed in 46% of the rats after a feeding period of 29 days; with 1·5% tyrosine, in 94% after an average of 19 days; with 2·0%, in 89% after an average of 14 days.

The frequency of alopecia and cheilitis was higher in the 1·5% group than in the group receiving 2·0%, because some rats receiving 2·0% tyrosine died within a few days before these signs appeared.

Pathological changes on the toes. After tyrosine feeding, the toes become swollen and reddened and the animals walk painfully. Later, dry squamous crusts appear (Pl. 1, fig. 3). The number of the toes affected rises in most cases as the dosage of tyrosine is increased.

Histological examination shows hyperkeratosis, acanthosis, oedema, diffuse cellular infiltration into the subcutaneous tissues and dilatation of the capillaries.

These changes were observed with 1% tyrosine in the diet in 4% (=2) of the animals after 15 and 29 days respectively; with 1·5% in 62% after an average of 23 days and with 2·0% in 64% after an average of 16 days.

Four animals of the group, which received 2·0% tyrosine, were further affected with an acute arthritis of the tibio-tarsal joints.

Alkaptonuria. Several days (3–8) after the beginning of tyrosine feeding homogentisic acid appears in the urine, which begins to turn brown.

Corresponding results were obtained in experiments with fifty white rats (24 ♂♂, 26 ♀♀; average weight 51 (45–60) g., average age 37 (33–52) days) fed on a synthetic diet richer in proteins and vitamins and receiving the above amounts of tyrosine per 100 g. body wt. per day.

The synthetic diet was similar to the diet used by Martin (1943) and had the following percentage composition: sugar 67, casein 18, oil 9, cod-liver oil 2, salts (McCollum) 4. It contained thiamin 1000 μg., riboflavin 2000 μg., nicotinic acid 20,000 μg., pyridoxine 200 μg., pantothenic acid 300 μg. per 100 g. Daily supplements of yeast (0·07 g.) and choline (0·003 g.) were given in addition to fresh water. The diet was supplied ad libitum. The tyrosine content of the proteins of the basal diet amounted to 900 mg. %.

With this diet of higher nutritional value, tyrosine had less effect on the rate of increase in weight and duration of life. The frequency and the speed with which the other signs of tyrosine poisoning appeared and their severity were unaltered.

Fig. 1. Keratitis after tyrosine feeding.

Fig. 2. Cheilitis after tyrosine feeding

Fig. 3. Signs on the toes after tyrosine feedin

Effect on oxygen consumption

Twenty-four rats (13 ♂♂, 11 ♀♀); average initial weight 59 (50–69) g.; average age 39 (34–55) days. Tyrosine was mixed with the diet in the following quantities: 1·0% (6 rats); 1·5% (6 rats); 2·0% (6 rats); control rats: 6.

The average values of each group are presented in Table 1, from which it will be seen that tyrosine added to the diet does not modify the oxygen consumption.

TABLE 1. Effect of tyrosine feeding on the consumption of oxygen by young white rats

Percentage of tyrosine in the basal ration	Oxygen consumption c.c./sq.m. surface area per 24 hr.				
	1st day	7th day	14th day	21st day	28th day
—	196	171	193	178	189
1·0	205	174	192	192	192
1·5	167	180	196	199*	—
2·0	193	174	177*	—	—

* Average from four rats.

Effect on morphology of the thyroid glands

No changes in the size or activity of the thyroid glands were observed in the case of twenty rats examined after feeding diets with 1·0 and 1·5% tyrosine from 15 to 40 days.

Effects on older white rats

Twenty-nine rats (15 ♂♂, 14 ♀♀); average initial weight 123 (100–150) g.; average age about 100 days. The effect of an addition of 2·0% tyrosine to the diet was investigated. The quantity of tyrosine (per 100 g. body wt.) which the rats received daily was between 0·57 and 0·59 g.

The data are presented in Table 2, in which the male and female rats are dealt with separately.

The chief results are as follows: (1) Among older rats the males are affected more severely than the females receiving the same amount of tyrosine. (2) When the results given in Table 2 are compared with those for younger rats it will be seen that older rats (both males and females) are less severely affected than younger ones receiving equivalent quantities of tyrosine. The loss of weight and the shortening of the survival period are less pronounced. The pathological symptoms appear later, and the greater survival time may be responsible for the higher frequency of some of the symptoms.

Effects on young white rats treated with various vitamins

Twenty rats (10 ♂♂, 10 ♀♀); average initial weight 70 (50–85) g.; average age 51 (40–70) days. The effects of cod-liver oil, riboflavin, pyridoxine and tyrosine were investigated in the following combinations: (1) tyrosine 1·5% + riboflavin (500 μg. daily, 6 rats); (2) tyrosine 1·5% + pyridoxine (1000 μg. daily, 6 rats); (3) tyrosine 1·0% + vitamin A (150 i.u. daily, 8 rats). The vitamin A was given orally, the B vitamins by subcutaneous injection.

These added vitamins did not affect the appearance of the pathological symptoms produced by tyrosine.

TABLE 2. Effects of tyrosine feeding on older white rats

Sex	Change in wt. (g.) Day 10	20	30	40	Deaths (% in 40 days)	Survival period (days)	Keratitis Rats affected (%)	Day of onset	Alopecia Rats affected (%)	Day of onset	Cheilitis Rats affected (%)	Day of onset	Symptoms on the toes Rats affected (%)	Day of onset
♂	− 3	− 14	− 21	− 26	60	35	100	15	66	23	100	16	93	24
♀	+ 5	− 6	− 12	− 20	7	—	100	17	35	24	70	31	35	30

TABLE 3. Effects of thiouracil and thyroxine on the occurrence of pathological changes after feeding large quantities of tyrosine to young white rats

	Change in wt. (g.) Day 10	20	30	40	Deaths (% in 40 days)	Survival period (days)	Keratitis Rats affected (%)	Day of onset	Alopecia Rats affected (%)	Day of onset	Cheilitis Rats affected (%)	Day of onset	Symptoms on the toes Rats affected (%)	Day of onset
Tyrosine 1·0%	+ 1·0	+ 2·5	+ 6·0	+10·0	17	36	78	19	49	21	46	29	4	—
+Thiouracil 0·1%	− 1·0	− 0·1	− 0·3	− 4·2	0	—	0	—	8	29	8	29	0	—
+Thyroxine 0·1 mg.	− 4·1	− 4·2	+ 1·7	+ 6·6	57	16	86	12	71	19	71	18	0	—
Tyrosine 1·5%	− 4·0	− 7·0	− 7·0	—	69	28	100	13	69	15	94	19	62	23
+Thiouracil 0·1%	− 3·8	− 3·9	− 5·8	− 4·0	29	29	93	18	14	18	21	28	0	—
+Thyroxine 0·1 mg.	−10·3	−14·5	−21·6	—	93	18	93	9	71	12	86	12	93	15
Tyrosine 2·0%	− 9·0	−15·5	−19·0	—	91	20	100	9	61	12	89	14	64	16
+Thiouracil 0·1%	− 3·0	− 3·2	− 5·6	—	58	24	100	16	19	20	50	17	8	(16)
+Thyroxine 0·1 mg.	−12·0	—	—	—	100	11	100	7	43	11	100	9	71	9
Thiouracil 0·1%	+ 1·0	+ 0·1	+ 0·4	+ 3·9										
Thyroxine 0·1 mg.	− 4·9	− 4·1	− 1·9	+ 1·9										
Basal diet	+ 4·0	+10·0	+15·0	+20·0										

Effects on young white rats treated with thiouracil and thyroxine

Ninety-two rats (45 ♂♂, 47 ♀♀); average initial weight 61 (40–100) g.; average age 41 (30–60) days. The effects of thiouracil, thyroxine and tyrosine were investigated in the following combinations: (1) tyrosine 1·0% + thiouracil 0·1% (12 rats); (2) tyrosine 1·5% + thiouracil 0·1% (14 rats); (3) tyrosine 2·0% + thiouracil 0·1% (12 rats); (4) tyrosine 1·0% + thyroxine 0·1 mg. (7 rats); (5) tyrosine 1·5% + thyroxine 0·1 mg. (14 rats); (6) tyrosine 2·0% + thyroxine 0·1 mg. (7 rats); (7) thiouracil 0·1% (12 rats); (8) thyroxine 0·1 mg. (14 rats). Thiouracil was mixed with the basal diet; thyroxine was given daily by subcutaneous injection.

The results are given in Table 3. For comparison, the results on untreated rats are included. It will be seen that thiouracil prolongs the survival time of rats treated with tyrosine and delays or even prevents the appearance of the syndrome. It lessens the loss of weight caused by an addition of 2·0% tyrosine to the diet (but thiouracil itself diminishes the gain in weight of young white rats). Thyroxine, on the other hand, increases the loss of weight and the frequency of pathological signs produced by tyrosine. It also hastens the appearance of these signs.

DISCUSSION

Pathological changes on the eyes, but not alopecia or cheilitis, have been described by Sullivan *et al.* (1932), Lillie (1932), Martin (1943), Martin & Hueper (1943) and Hueper & Martin (1943). Moreover, though they observed diffuse erythema and swelling of the extremities, they did not describe the hyperkeratosis, acanthosis and signs of acute inflammation in the subcutaneous tissues of the toes reported here. The reason for these differences may be found in the different basal diets, the amounts of tyrosine consumed or in racial differences among the rats. Schroer (1938), Carrié (1939) and Krock (1939) state that tyrosine feeding in rabbits increases liability to inflammation of the skin, and that this phenomenon can be prevented by administration of an adrenal cortex preparation. Chamorro (1946) has recently observed the appearance of an arthritis in the tibio-tarsal joints in rats whose adrenals have been removed.

It has long been known that the addition of tyrosine or phenylalanine to the diet produces alkaptonuria. This was demonstrated in white rats by Papageorge & Lewis (1938), Fölling & Closs (1938), Butts, Dunn & Hallman (1938), Closs & Braaten (1941), Butts, Sinnhuber & Dunn (1941), Lanyar (1942b) and Abbott & Salmon (1943), in white mice by Lanyar (1942a) and in guinea-pigs by Sealock & Silberstein (1939, 1940), Sealock, Ziegler & Driver (1939), Sealock, Parkinson & Basinski (1941) and Sealock (1942). The amount of tyrosine which produces alkaptonuria in rats can also cause morphological changes. This amount was found to be from 0·2 to 0·3 g./100 g. body wt./day, given for several days.

It is of interest that older male rats are more severely affected than females of the same age. Epithelial atrophy and spermatid giant cell formation in the

testes of rats after tyrosine feeding were demonstrated by Hueper & Martin (1943). An explanation for this sex difference cannot be given; Fishman & Artom (1942), however, have observed the same phenomenon in investigations with *dl*-serine.

According to the present findings and those of Martin (1943), Martin & Hueper (1943) and Hueper & Martin (1943), deficiencies in vitamins or in protein in the basal diet cannot be held responsible for the production of the pathological symptoms by tyrosine. Martin & Hueper showed that an additional amount of yeast or ascorbic acid had no effect on the severity of the symptoms; our investigations demonstrated that neither vitamin A, riboflavin nor pyridoxine can alleviate the syndrome of tyrosine poisoning. The keratitis cannot have been produced by a deficiency of tryptophan, lysine or methionine, as was described by Totter & Day (1942), Albanese & Buschke (1942), Albanese (1945) and Sydenstriker, Hall, Hock & Pund (1946). Our findings that the oxygen consumption is not changed by the addition of tyrosine to the diet, are contrary to those of Delcourt-Bernard (1937), who demonstrated an increase of the basal metabolism in rabbits after tyrosine administration, and of Oehme (1939, 1940, 1941), who found that in guinea-pigs tyrosine decreased the oxygen consumption. The results of the latter author concerned experiments on three animals; the decrease did not exceed 13%.

There is also no morphological change in the thyroid gland. The slight increase of the activity of the glands pointed out by Hueper & Martin (1943) may possibly be explained as a normal variation of activity. It is believed that thiouracil prevents the synthesis of the hormone in the thyroid gland, while thyroxine produces some of the symptoms of thyrotoxicosis. The investigations made with thiouracil and thyroxine therefore indicate that the appearance of the symptoms of tyrosine poisoning may depend to a large extent on the degree of activity of the thyroid gland. Abelin (1935a, 1935b), Delcourt-Bernard (1936) and Oehme (1936) found, on the other hand, that tyrosine is capable of mitigating some of the symptoms of experimental hyperthyroidism.

SUMMARY

1. Addition of *l*-tyrosine to the diet of young white rats produces a loss of weight, a shortening of life, alkaptonuria and the appearance of a characteristic syndrome (keratitis and conjunctivitis; alopecia; cheilitis; hyperkeratosis, acanthosis and inflammatory processes on the toes).

2. The smallest amount of *l*-tyrosine administered for several days which is capable of producing these changes is between 0·2 and 0·3 g./100 g. body wt./day. The loss of weight, the shortening of life, the frequency and speed with which the syndrome appears within a group of rats and the severity of the symptoms depend on the quantities of tyrosine consumed.

3. Young rats are more severely affected than older ones. Among older rats the effect of tyrosine is more marked in the males than in the females.

4. Deficiencies of protein, vitamin A, riboflavin or pyridoxine cannot be held responsible for the production of these pathological changes.

5. The activity of the thyroid gland is not modified by tyrosine feeding.

6. Thiouracil diminishes the loss of weight, prolongs the life and prevents or delays the appearance of the symptoms of tyrosine poisoning. Thyroxine has an opposite effect.

The corneas of a number of rats were observed by means of a keratoscope at regular intervals over a period of 25 days. We wish to express our gratitude to Dr Brueckner (University Hospital, Basle) for these investigations.

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RIGOR MORTIS AND ADENOSINETRIPHOSPHATE

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Erdös (1943), working in Szent-Györgyi's laboratory, has shown that the destruction of adenosinetriphosphate (ATP) and the hardening of rabbit's muscle follow parallel courses during the development of rigor mortis. Taking into account the known importance of ATP in muscular contraction, the fact that myosin itself can apparently act as an adenosine triphosphatase (Engelhardt & Ljubimova, 1939; Bailey, 1942), and the effect of ATP in vitro on myosin (or actomyosin) sols and artificial fibres (Dainty, Kleinzeller, Lawrence, Miall, Needham, Needham & Shen, 1944; Szent-Györgyi, 1945), Erdös and Szent-Györgyi conclude that lack of ATP is in fact responsible for the stiffening of the muscle. Although lactic acid formation generally accompanies the development of rigor in muscle, Claude Bernard (1877) and later Hoet & Marks (1926) demonstrated that rigor could occur in the complete absence of acid production. Hoet & Marks therefore suggested that the factor providing common ground between the acid and alkaline types of rigor was the disappearance of hexose phosphate from the muscle. Smith (1930) pointed out that, while the facts clearly required the assumption of a third change to which both stiffening and lactic acid were related, many further possibilities of chemical change existed.

The present work confirms that breakdown of ATP is correlated with the onset and development of rigor in normal animals. It extends Erdös's observations to animals with depleted reserves of glycogen, and shows that in these cases also there is a relation between stiffening and disappearance of ATP. The same relation does not hold between stiffening and lactic acid production even when the acid production is of normal magnitude, but the coincidence observed by Bate-Smith (1939), between the initiation of rigor and the attainment of a pH in the neighbourhood of 6·2, is confirmed. A mechanism is suggested for this coincidence through the interaction of ATP breakdown and the glycolytic cycle in muscle. It would appear that the breakdown of ATP is, in fact, the 'third change' which in 1930 remained to be identified.

METHODS

Preparation and extraction of muscle. The rabbits used in these experiments were killed by a blow on the nape of the neck and bled thoroughly after decapitation. The psoas major muscles were then removed as rapidly as possible, one being used for the determination of adenyl-polyphosphate phosphorus (i.e. the labile phosphorus of ATP and ADP) and the other for measurement of the modulus of elasticity by the method of Bate-Smith (1939). It has been shown by Bate-Smith (1939) that the development of rigor takes place at an equal rate in the left and right psoas muscles, both in 'normal' rigor and in 'alkaline' rigor following insulin treatment.

For the analysis of the phosphorus compounds, samples weighing 1–2 g. were cut progressively from the proximal end of the muscle at the times shown in the tables, the first sample being cut within 7 min. of death. The samples were then plunged into 5 c.c. ice-cold 10% trichloroacetic acid (TCA) in a mortar, and ground to a fine pulp with sand. The pulp was washed on a filter with 4×5 c.c. 5% TCA, and the filtrate collected in a 50 c.c. volumetric flask. The pH was then adjusted to 8·0 by the addition of N-NaOH, and the volume made up to 50 c.c.

Estimation of phosphorus content. The 'inorganic-P' content was estimated on 2 c.c. aliquots of each extract by the colorimetric method of Allen (1940), the final volume of the coloured solution being 25 c.c. in each case. The colour intensity was measured after 25 min. on a Spekker photometer, using a red filter. In this way all the photocreatine present is hydrolysed to inorganic phosphate and creatine, and the so-called 'inorganic-P' therefore includes phosphocreatine-P.

The '7 min.-P' was also estimated on 2 c.c. aliquots, after hydrolysis in N-HCl for 7 min. at 100°C.

The 'total P' was estimated on 1 c.c. aliquots after hydrolysis with perchloric acid by the method of Allen (1940).

The adenyl-polyphosphate phosphorus content is given approximately by difference between the '7-min.-P' and 'inorganic P'. The former, however, may contain a small amount of phosphate arising from the partial breakdown of other phosphate esters, most of which require more than 2 hr. for complete hydrolysis under these conditions. During the first 14 min. the hydrolysis curve of these compounds is virtually a straight line, and hence, by carrying out a 14 min. hydrolysis in N-HCl, an estimate can be obtained of the non-polyphosphate phosphorus appearing in the first 7 min. The polyphosphate values in the tables and figures have been corrected to take account of this.

Estimation of lactic acid content and pH values. The lactic acid content was estimated on 30 c.c. aliquots of the extracts by the method of Friedemann & Graeser (1933), after treatment with copper-lime mixture to remove reducing compounds.

The pH of the samples was estimated by means of the glass-electrode on 1–2 g. of minced muscle, moistened with M/200-sodium iodoacetate. Owing to the small amount of muscle available it was not possible to measure the pH values side by side with the phosphorus values, and the stated pH values are therefore calculated from the final pH, the lactic acid values and the buffering capacity. The latter was measured by titration with 0·05 N-HCl or -NaOH, of the same samples as those used for determination of pH. The pH values so calculated are correct but for an amount due to escape of CO_2 which makes them too high by $\not> 0·2$ pH unit in the early post-mortem period. Thus the pH value calculated for muscle completely free from lactic acid is always about 7·6, whereas the value is more probably 7·4 (cf. Bate-Smith, 1938).

Variations in composition of samples taken simultaneously and errors of determinations. In order to estimate the magnitude of errors due to these factors a rabbit was killed and one psoas muscle removed immediately. This was then cut into six portions, weighing 1–2 g. each of which was plunged immediately into 10% trichloroacetic acid. The samples were immediately extracted and the phosphorus content estimated. The mean error of estimation of the inorganic, total '7-min.' and total P values amounted to less than 2% of the values in each case. On the other hand, the error in the polyphosphate-P values, obtained by difference, was found to be at least $\pm 5\%$, becoming greater as the values become smaller, thus effectively limiting the reliability of the method to estimation of values not less than 0·05 mg. polyphosphate-P/g.

<div align="center">RESULTS</div>

The results of four experiments in which the rates of onset and development of rigor were very different are given in Table 1. The phosphate values are expressed as mg./g. of fresh muscle. The unidentified phosphorus content (UP), which is the difference between the polyphosphate + inorganic phosphorus and the total, is also given in the table. This fraction contains mainly the phosphorus of adenylic acid, of hexose-1-, -6- and -di-phosphates and triose-phosphate. The lactic acid values are expressed as mg. lactic acid per g. of muscle.

In Figs. 1 and 2 the polyphosphate-P values, the pH and the modulus (E) values are plotted against time. The polyphosphate-P remaining at any time is plotted as mg. P/g. of muscle, and the modulus as a percentage of the final modulus attained.

TABLE 1. Changes in the phosphorus compounds during
development of rigor mortis in rabbit muscle

			Phosphorus and lactic acid values				Modulus	
Time after death (min.)	In-organic P (mg./g.)	Poly-phos-phate P) (mg./g.)	Uniden-tified P (mg./g.)	Total P (mg./g.)	Lactic acid (mg./g.)	pH (calc.)	Time after death (min.)	E g./cm.²
Exp. 5. 3 kg. female.								
Normal. Well fed. Intra-								
peritoneal injections of								
glucose								
4	0·84	0·33	0·60	1·77	3·25	6·91	22	610
43	0·85	0·37	0·56	1·78	2·85	6·99	128	715
113	0·91	0·32	0·58	1·81	3·85	6·78	240	810
236	0·82	0·26	0·57	1·65	4·90	6·55	375	4600
368	0·95	0·11	0·61	1·67	6·90	6·08	540	4600
556	1·11	0·08	0·56	1·75	8·15	5·74	600	4600
1440	1·33	0·00	0·41	1·74	8·75	5·58	1440	5000
Exp. 6. 2·5 kg. female.								
Starved 40 hr. Injected								
with insulin at 8.15 a.m.								
and at intervals thereafter.								
Died at 5.40 p.m. Total								
dose of insulin = 121 units								
4	1·24	0·36	0·19	1·79	0·35	—	15	2440
16	1·33	0·24	0·22	1·79	0·165	—	44	6600
33	1·45	0·15	0·18	1·78	0·10	—	83	8000
78	1·59	0·17	0·14	1·90	0·10	7·26	1440	8000
963	1·63	0·07	0·09	1·79	0·55	7·25		
Exp. 8. 2 kg. female.								
Normal. Fed. Intraperi-								
toneal injections of glucose								
5	1·16	0·34	0·50	2·00	3·80	6·86	23	900
55	1·46	0·15	0·41	2·02	5·97	6·45	37	1800
61	1·55	0·16	0·41	2·12	5·45	6·54	56	3000
82	1·61	0·11	0·39	2·11	6·09	6·43	73	3650
130	1·58	0·11	0·39	2·08	6·75	6·28	91	4050
1440	1·91	0·01	0·19	2·11	8·20	5·91	110	4250
330	—	—	—	—	—	6·22	360	4700
							1440	4900
Exp. 9. 2·5 kg. male.								
Normal. Well fed								
4	0·71	0·47	0·69	1·89	3·96	6·83	25	890
229	0·93	0·36	0·71	2·00	6·25	6·43	93	1000
304	0·85	0·23	0·94	2·02	6·65	6·35	260	1100
329	0·93	0·17	0·90	2·00	7·44	6·20	337	1950
344	0·90	0 17	0·91	1·98	7·70	6·14	347	3100
371	0·86	0·21	0·86	1·93	8·05	6·06	364	4450
444	0·96	0·14	0·92	2·02	8·75	5·90	382	6100
							398	7000
							1440	7400

<div align="center">DISCUSSION</div>

The outstanding feature of all the experiments, not only of the four typical examples quoted but also of others which for the sake of brevity have not been detailed, is the close correspondence between the rate of disappearance of the adenyl-polyphosphate-P fraction and the rate of increase of the modulus of elasticity. In the three experiments in which lactic acid production occurred,
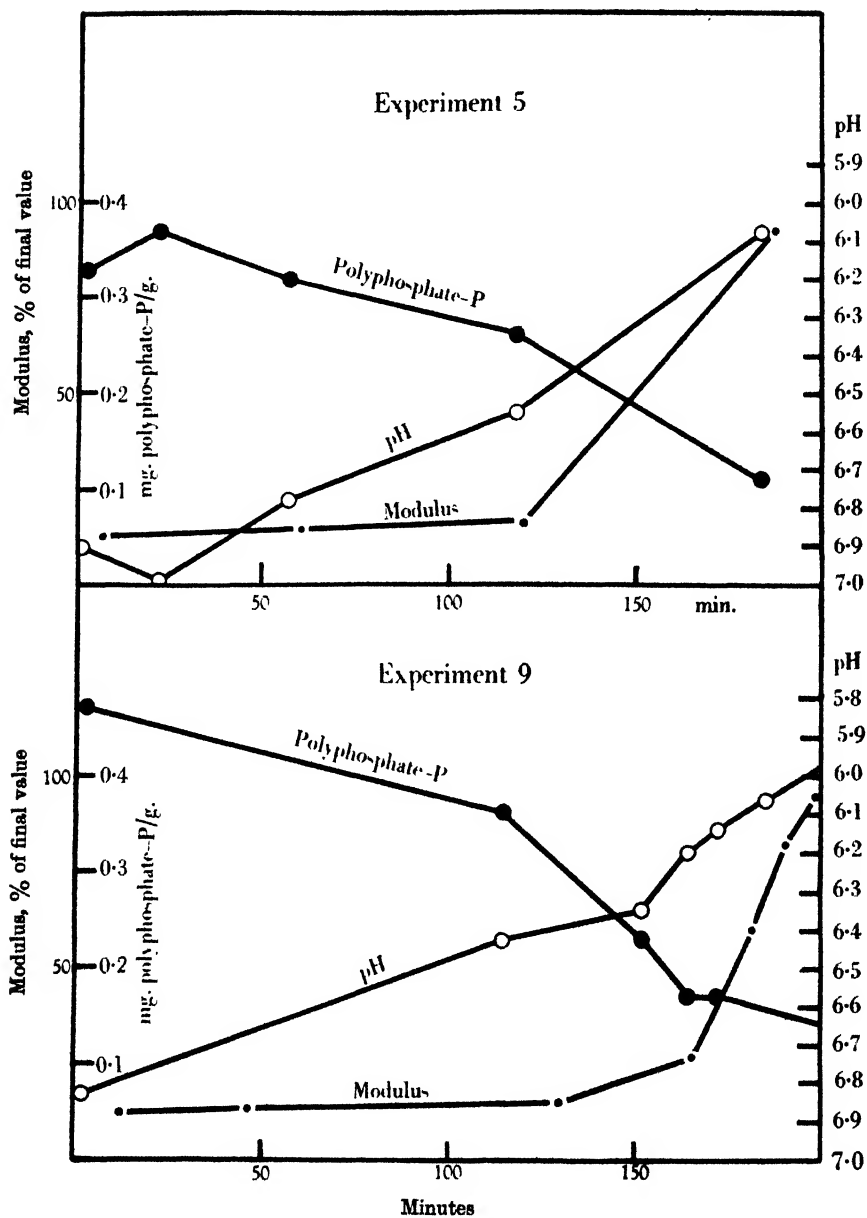
Fig. 1. The modulus, polyphosphate-P and pH values of rabbit psoas muscle during development of rigor mortis. Exp. 5, normal rabbit; ultimate pH 5·58. Exp. 9, normal rabbit; ultimate pH 5·60.
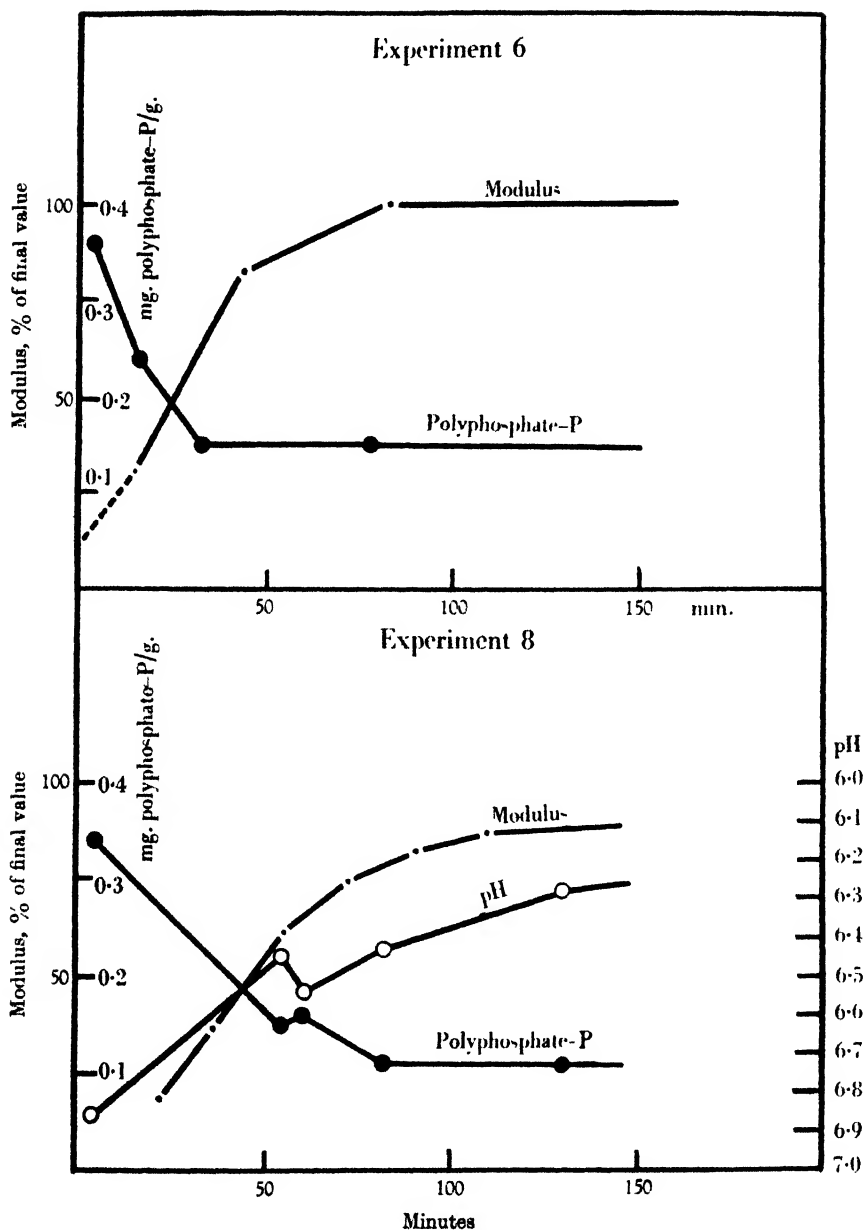
Fig. 2. The modulus, polyphosphate-P and pH values of rabbit psoas muscle during development of rigor mortis. Exp. 6, insulinized rabbit; ultimate pH 7·25. Exp. 8, normal rabbit; ultimate pH 5·91.

and in which the rapid stage of rigor was delayed, an increase in rate of break-down of polyphosphate coincided with the rapid onset of rigor. In the fourth experiment (Exp. 6), in which there was no production of lactic acid, no delay occurred in either the onset of the rapid stage of rigor or the rapid breakdown of polyphosphate.

The production of lactic acid when it does occur, is continuous from the moment the first sample is taken and does not as a rule cease when rigor is completed. There is, however, some degree of acceleration during the rapid stage of rigor which is probably secondary to the rapid breakdown of poly-phosphate at this stage. There is every reason to suppose, therefore, that the process of stiffening is directly connected with the decrease in the polyphosphate fraction, and is in fact, as Erdös (1943) deduced, attributable to the disap-pearance of ATP from the system.

The plan of our experiments did not permit of determinations of ATP; the behaviour of ATP and of ADP must therefore be assumed from the evidence available.

The following enzymes have been claimed to be present in muscle: an ATP-ase, associated with myosin (Engelhardt & Ljubimova, 1939) and active in the breakdown of ATP to ADP at pH 7 and more alkaline reactions, and myokinase, which catalyses the breakdown of ADP to adenylic acid, possibly with some resynthesis of ATP (Kalckar, 1943). Sakov (1941) has further isolated a transesterifying enzyme, with maximum activity at pH 7, which catalyses the transference of phosphate from ATP to fructose-6-phosphate (Neuberg ester) and a mineralizing enzyme which catalyses the production of inorganic phos-phate from ATP and ADP at a pH optimum of 6, the reaction proceeding feebly at pH 7. In experiments with dialysed muscle juice to which ATP and fructose-6-phosphate were added, Sakov found that at pH 7 the main reaction was trans-esterification while at pH 6·0 inorganic phosphate production predominated.

Besides these phosphorylases and phosphatases, it may be assumed that under the conditions of our experiments the anaerobic glycolytic cycle will be operative. In this cycle, the breakdown of glycogen to lactic acid (2 mol.) is coupled with resynthesis of 3–4 mol. of ATP from ADP or adenylic acid (Lipmann, 1941; Needham, 1942).

From this it is evident that the conditions may be very different, depending on whether glycolysis does or does not occur. When the glycolytic cycle is unable to operate owing to total absence of glycogen, as in Exp. 6, the break-down of polyphosphate, unaccompanied by any resynthesis, is rapid from the moment of death, but slows down markedly when about 50% of the labile P has disappeared. Even after 24 hr. 25% of the original polyphosphate-P was still present in the muscle. Further experiments are required to determine the precise nature of this polyphosphate-P.

On the other hand, when glycolysis is fully operating, as in Exps. 5, 8 and 9,

the first stages of the polyphosphate curve will represent a balance between breakdown and synthesis. Even at this stage, however, the conditions favour breakdown, which proceeds slowly for about 4 hr. in Exps. 5 and 9, but rapidly from the outset in Exp. 8. In Exps. 5 and 9 the rate of breakdown increases fivefold after this initial lag period. This increase begins when the pH is about 6·5. It is during the period of breakdown of polyphosphate, not during the preceding lag period, that the significant change in modulus occurs. If resynthesis of ATP is proceeding this change in rate of net breakdown might be caused by a decrease in rate of resynthesis or by an increase in rate of total breakdown. However, the rate of production of lactic acid, and hence of resynthesis, does not decrease, but rather increases, during this period. The net increase in breakdown must, therefore, be due to the increase in gross breakdown. It is permissible to attribute this to the activity of the mineralizing enzyme described by Sakov, which becomes greater as pH 6·0 is approached. If at this stage the glycogen in the muscle is exhausted, so that the pH remains in the neighbourhood of 6·0, as in Exp. 8, the breakdown of polyphosphate appears to proceed rapidly to completion, but if the pH falls further (Exps. 5 and 9), the reaction slows down and appreciable amounts of polyphosphate may still be present after 24 hr.

Thus, although it is clear that destruction of glycogen, leading to production of lactic acid and fall in pH, is not directly responsible for the increase in modulus, the moment at which the modulus begins to increase and its rate of increase are both clearly dependent upon the pH of the system and the prevailing rate of glycolysis, because these are also the factors on which the rate of destruction of ATP depends. As we have seen, it is this destruction of ATP and this alone with which the onset of rigor can be associated, whether or not accompanied by glycolysis.

The results substantiate Erdös's conclusions. As we have seen, however, the circumstances of rigor are more complicated than would be supposed from the linear relations which he depicts both for the breakdown of ATP and for the onset of stiffening with time after death.

Factors determining the time-course of rigor. There is considerable variation in the time after death at which rigor mortis sets in. We have observed that in animals which are relatively passive before stunning, the delay before the onset of rigor is longer than in animals which struggle violently, and this delay becomes still longer in narcotized animals (Bate-Smith, 1939). It seems likely that two factors may be responsible for the observed variations: the actual production of lactic acid in the muscles as a result of struggling; and a subsequent and sustained increase in the rate of glycolysis. Any factor which has an effect on the metabolic rate of resting muscle can, in fact, be assumed to influence the rate of production of lactic acid immediately post mortem, and thereby affect the period of delay before rigor sets in.

Shortening during rigor. Shortening occurs only when rigor develops at a pH higher than 6·2 and is not a normal concomitant of rigor. It is probable that below this pH the muscle is no longer capable of shortening when stimulated, so that the stimulus (whatever it may be) that causes contraction when rigor sets in above pH 6·5 may always be present at the moment rigor sets in whether a response follows or not. In isolated muscles it is observed that, when rigor is precipitate the shortening is greater and accompanied by greater development of tension. An extreme instance of this is shown in Fig. 3, which is a reproduction of the tracing of the psoas muscle in Exp. 8, illustrating the behaviour on application and removal of a load of 50 g./cm.² immediately after death. The muscle was then at pH 6·7, and rapidly going into rigor. A total shortening of 16 % occurred. The tension developed by this shortening, calculated from the magnitude of the response to the applied load, is of the order of 250 g./cm.²,
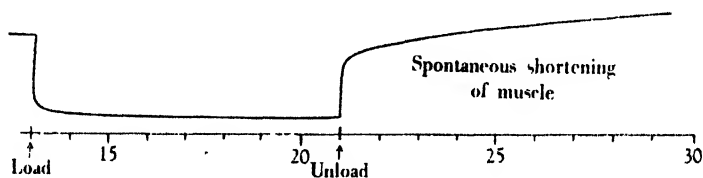
Fig. 3. Form of extension and recovery of rabbit psoas muscle resulting from application and removal of load, showing both the immediate and delayed phases of deformation and the effect of shortening on recovery curve as rigor is initiated. (Redrawn from kymograph tracing of Exp. 8.)

i.e. about 1/20th only of the maximum force that the muscles are capable of developing (cf. Haxton, 1944). It is to be remarked again that this represents an unusual degree of shortening, corresponding to the unusually high pH at which rigor sets in.

Only rarely in practice, at least at the slaughter house, does rigor take a course other than that of Exps. 5 and 9, since the glycogen content is usually sufficient to lower the pH to 5·6 or below. The rabbits available for the present work, in contrast to those used before the war, tended to have an abnormally high ultimate pH, due no doubt to the low level of feeding which it had been necessary to maintain during their rearing. After a fortnight's feeding with a supplement of starch the glycogen content improved to the extent indicated in Exps. 5 and 9.

SUMMARY

1. The stiffening of mammalian muscle during the onset of rigor mortis is correlated with a decrease in adenyl polyphosphates which is specifically interpreted as a decrease in adenosine triphosphate. Thus the earlier observation of Erdös (1943) is confirmed.

2. The correlation is observed both in normal ('acid') and in 'alkaline' rigor. In the former, acid production also runs parallel. An explanation of this

secondary correlation, and also of the normal lag period before onset of rigor, is put forward in terms of the variation with pH of the activity of the poly-phosphatases known to be present in muscle.

3. Shortening occurs only if the pH of the muscle is greater than about 6·2 when stiffening sets in. This pH is unusual in animals with a normal reserve of glycogen. The force associated with shortening, when it occurs, is small in comparison with the absolute force developed during voluntary contraction.

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THE EFFECT OF SEAT POSITION ON THE EFFICIENCY OF BICYCLE PEDALLING

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Different aspects of cycling illustrate well the various physiological factors that determine the efficiency of utilization of muscle force after this has once been developed by the muscles. For example, the effects of different rates of pedalling have been considered by Benedict & Cathcart (1913), by Hansen (1927), who also considered the effects of different pedal loads, and, more recently, by Garry & Wishart (1931) who demonstrated the existence of a most efficient pedalling speed of 52 r.p.m.

The experiments to be described were undertaken to determine the effect of different seat positions; seat position determines the leg-joint angles which finally control the thrust available from the force produced by the muscles.

The increase in force exertable with knee-joint extension explains the well-known cycling requirement of adjusting the saddle to allow full leg-extension. The findings described in a previous paper (Hugh-Jones, 1947) showed that especially large pushes could be exerted on a foot-pedal by using the leg as a mechanical toggle between a pedal and the back-rest of a carefully adjusted seat. It was, therefore, thought possible that efficiency of cycling might be greater than that obtainable when using a correctly adjusted saddle in the normal cycling position, if the operator used a seat with a back-rest placed in a position both further back and lower than the normal saddle, so allowing full toggle-action with the legs.

The work was started in relation to stationary bicycle pedalling as a means of utilizing human muscular power.

APPARATUS AND METHOD

The standard type of bicycle ergometer, with a 70 lb. fly-wheel, was used. The load on the brake-band was measured, to within 1 %, by taking the difference reading between the two balances; particular care was taken to avoid error from stiction in the system that carried the cords from the brake-band to the balances: only two pulleys, of 5 in. diameter, were included in this system and these were mounted on ball-races lubricated with upper-cylinder lubricant.

A frame was arranged to permit a seat, cut away like a saddle but carrying an adjustable back-rest, to be rigidly fixed in any desired position on the arc of a circle with centre at the pedal axle; the radius of the arc was determined by the effective leg-length of individual subjects.

The Douglas-Haldane method was employed to estimate energy expenditure. The Haldane apparatus was checked before samples were analysed on any day. The readings from this procedure gave a mean O_2 percentage for atmospheric air of 20.92 ± 0.003 (s.e. of mean of 85 observations recorded on different days).

The gas samples for analysis were collected from the Douglas bags into Brodie sampling bottles and analysed in duplicate; the data obtained from the two analyses showed good agreement and their mean value is used in the calculations which follow.

Two males, aged 28 years, of weights 168 and 149 lb., and of heights of 71 and 68 in. respectively, acted as subjects.

Experiments were always started at the same time in the mornings. At every load in 'step-up' experiments (see later), and for each cycling position in the other experiments, the subject pedalled, in time with a metronome, at 52 r.p.m. with the mouthpiece for breathing in position but with the two-way tap open to the atmosphere, for exactly 5 min. This time was chosen as a result of preliminary experiments to determine the minimum time necessary to ensure that a 'steady-state' had been reached. Expired air was then collected over a sampling period, lasting about 2 min., the two-way tap being turned on and off at the end of an inspiration concurrently with a stop-watch which recorded the exact time of sampling. Inspiratory alveolar-air samples were taken, when required, by means of a modified Haldane sampling apparatus (Peters & Van Slyke, 1932) as soon as the collection of expired air was finished.

The cycling was done without holding the handle-bars and without 'ankling' with the pedals, since the advantage gained by such procedures would be both variable and indeterminable.

The experiments lasted over six months. After the first month, which was occupied with the preliminary experiments, further training effects were not noted in the subjects who were in fair training before the start of the experiments. In the main series, the order of experiments was randomized in relation to load and seat position and it is hoped that this minimized any effects of training and day-to-day variation that may have occurred in a subject.

RESULTS

The oxygen requirement is a measure of the energy needed to perform a given amount of mechanical work provided the work can be performed in a 'steady-state' (Hill, Long & Lupton, 1924); this measure was considered to be the best means of comparing the different seat positions by finding for each the effort required to perform a constant amount of mechanical work.

As the preliminary experiments had shown that the differences in oxygen requirement were likely to be small, it seemed important that the constant amount of external work for each subject should be as large as possible and yet be performable in a steady state, that is, the work level should not exceed the 'crest-load'. Moreover, the results for each subject would be better related if the cycling positions were compared at a comparable load for each individual, and for this purpose also, a load just below his crest-load is suitable. The crest-load, therefore, first had to be found.

Determination of the 'crest-load'. Briggs (1920), who introduced the term 'crest-load' as the highest load which could be maintained in a steady state, originally used the load at which a fall in the proportion of carbon dioxide in the expired air occurred; Hill & Lupton (1923) and, later, Bock, Vancaulaert,
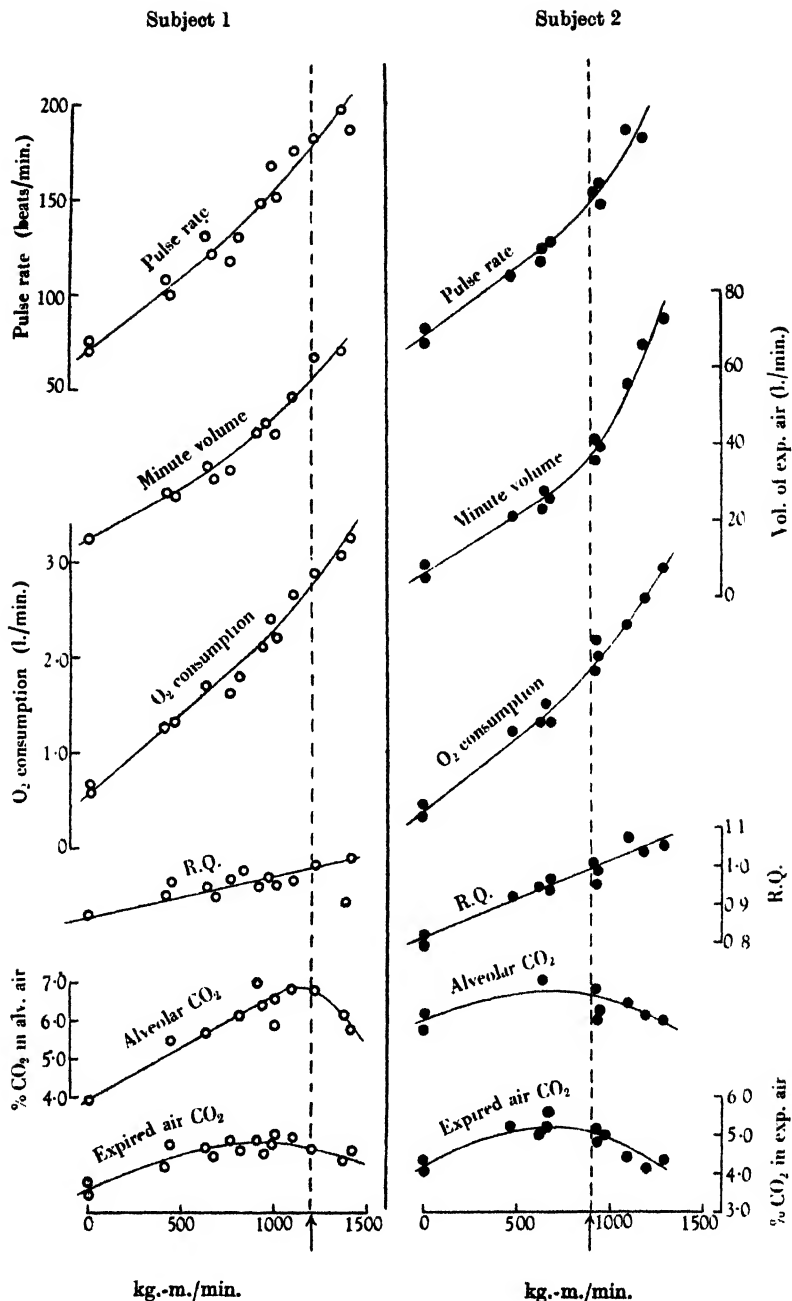
Fig. 1. 'Step-up' experiments to determine the approximate 'crest-load' of the two subjects
The approximate 'crest-load' indicated by the arrow is determined from the response of the
different physiological functions (ordinates) to increasing mechanical work (abscissae).

Dill, Folling & Hurxthal (1928) showed that the R.Q. passed unity at this load; Schneider (1931) used the load at which heart rate and oxygen consumption failed to show a continued linear increase and at which total ventilation increased excessively. In the present work as many criteria as possible were used, according to the 'step-up' method of experimentation described by Taylor (1941).

The results for cycling with the saddle in the normal position are shown in Fig. 1.

The points on the graphs represent results from five experiments performed on different days. It will be seen that, from the different criteria mentioned (the R.Q. exceeding unity; the minute-volume of air and the oxygen consumption ceasing to increase linearly with load; and the percentage of CO_2 in alveolar air reaching a maximum), the crest-load of subject 1 was approximately 1200 kg.-m./min. while that of subject 2 was only 900 kg.-m./min. approximately.

It is interesting to note that in these two subjects, the rate of increase in oxygen consumption for increasing work is augmented above the crest-load and not diminished as occurred in some of Schneider's subjects. Moreover, the peak of CO_2 in the expired air occurred before the crest-load as judged on the other criteria. This finding does not agree with the results of Briggs but confirms those of Schneider. The results support Taylor's conclusions that these different functions behave in a variable fashion after the limit of work that can be performed in a steady state is reached.

Comparison of the different seat positions. The seat with a back-rest was used. The latter, a small padded rectangular plate of about 6 × 4 in., was adjusted with its centre 6 in. above the seat so that the pelvis was directly supported. The position of the seat on different parts of the arc about the pedal axle was measured by the angle formed between the perpendicular and the line joining the base of the seat-back to the pedal axle. On this basis, the normal cycling saddle makes an angle of 26°.

The load on the ergometer was kept constant, as far as possible, so as to adjust output of each subject to approximately 100 kg.-m./min. below his crest-load. Actually, owing to small changes in brake-band friction, the load varied by just under 2 %; that is, it was always within 1110 ± 20 and 805 ± 15 kg.-m./min., respectively, for the two subjects.

The results are shown graphically in Fig. 2. Here the ordinates represent the mean amount of oxygen consumed for the constant amount of external work. This is given as the former divided by the latter (converted to Calories for convenience) in order to correct for the small day-to-day variation in work, discussed above; this correction is justified as it is small and the relation between the two variables is known to be linear below the crest-load. The abscissae represent the seven seat positions used, given in degrees of rotation

about the pedal axle as mentioned above. The limits shown for the mean oxygen consumption represent ± twice the standard error. This is calculated from the number of readings given below each point; these readings were obtained on different days.
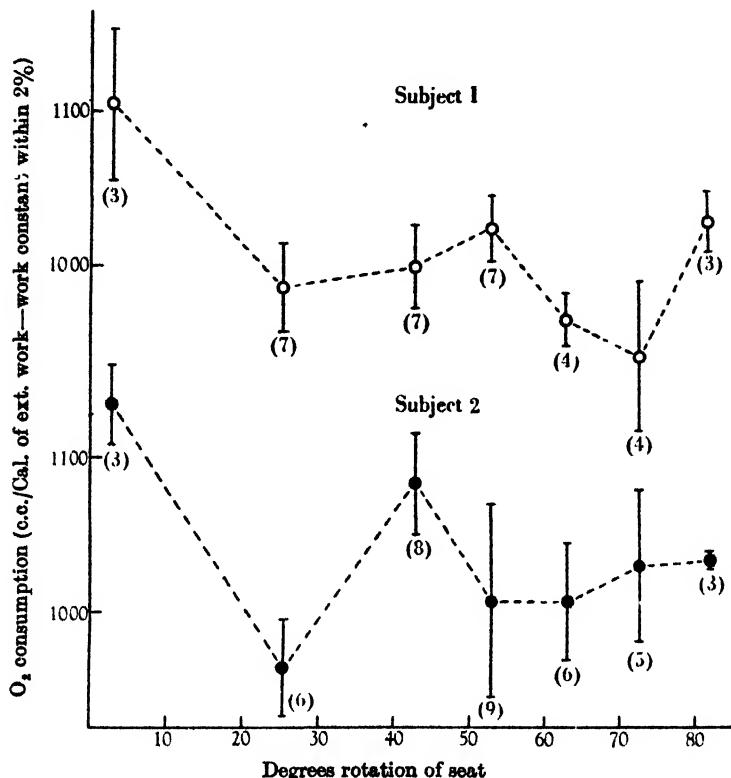
Fig. 2. Oxygen used (ordinates) for performing a constant amount of work with the seat in different positions on the arc about the pedal axle (abscissae). Circles: subject 1; Dots: subject 2.

It will be seen that, for both subjects, the amount of oxygen consumed falls significantly from the 3° position to a minimum at 26° which is the normal cycling position. It tends to rise again as the seat is moved further back, to fall again to another minimum somewhere about 63° (± 10°), and finally to rise as the seat, in the 82° position, approaches the horizontal through the pedal axle. These results indicate that two positions only are profitable for cycling; one is the normal saddle position, the other, needs a seat with back-rest, and occurs at about 63° (± 10°). It is interesting to note that the latter corresponds with the position adopted in the French 'Velocar' racing bicycle. Between the two positions there appeared no significant difference, so a further series of experiments was made with these two optimum positions only.

Comparison of the two 'optimum' seat positions found. The normal cycling position was compared with the 63° seat position, with the back-rest for the latter at 6 and 11 in. above seat level. 'Step-up' experiments were used involving three different loads, one of which was above the crest-load of the subject. By this means, again by measuring oxygen consumption, it was
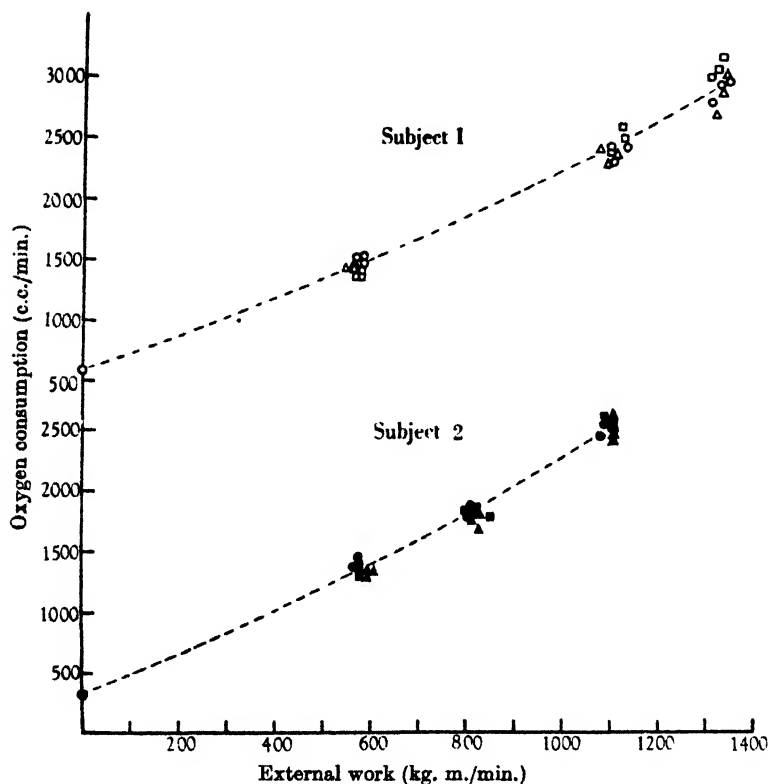
Fig. 3. Comparison of the two 'optimum' cycling positions found in terms of the oxygen consumed (ordinates) at different levels of external mechanical work (abscissae). (Circles = normal cycling position; triangles and squares = alternative position with low and high back-rest, respectively. Open symbols, subject 1; black symbols, subject 2.)

hoped to detect any difference between the positions. If such a difference existed it would, from the previous results, be very small, but this method might allow two criteria of difference: possibly different loads for the crest-load, and, more likely, divergence of the straight lines joining the points for oxygen consumption at different loads up to the crest-load.

The results are shown graphically in Fig. 3. (The 'no-load' O_2 consumption was not redetermined. The value indicated for it in Fig. 3 is that found in the previous 'step-up' experiments described above.)

From Fig. 3 it will be appreciated that the results might seem to indicate a very slight advantage of the 63° position with a low back-rest over the normal cycling position, but the differences are not statistically significant. It can only be concluded that, with the methods used and number of results obtained, no advantage could be detected in energy cost for 'back-rest cycling' over the more usual position, though it appears to be a practicable alternative to the latter.

DISCUSSION

It seems that the results shown in Fig. 2 may be explained by the operation of two effects: the first is gravitational and helps most when the legs of the operator are vertically above the pedals; the second is mechanical toggle-action between the seat back-rest and pedals and this helps most (as discussed in the previous paper, Hugh-Jones, 1947) when the pedals are on the same horizontal level as the seat. Thus, in Fig. 3, the forward 3° position is less advantageous than the normal cycling position because the cyclist's weight cannot be fully exerted on the pedals which consequently feel subjectively 'too far under the seat'; behind the normal cycling position 'static effort' (Bedford, Vernon & Warner, 1933), from upholding the legs, progressively decreases efficiency as the seat is moved backwards round the arc about the pedals. At the same time, after about the 53° position, the back-rest, of which no use can be made before, increasingly exerts its beneficial influence. It outweighs the adverse effect from static effort at about the 63° position, but then ceases to do so as the seat further approaches the horizontal passing through the pedal axle, because its effect appears to increase less rapidly than that of static effort.

From the aspect of the practical application of the above results two points are interesting: first, in the evolution of the modern bicycle, as given by Sharp (1896), the seat position accepted to-day has been adopted after trial of positions both just in front of, and just behind it; secondly, in view of the difficulty in detecting any significant difference between the usual position and the low back-rest position, the 'Velocar', which employed approximately this second alternative position with a back-rest, gained the kilometre (flying start) and the 50 kilometre (standing start) 'records libres' in recent world cycling records (Union Cycliste Internationale, 1939). The conventional machines, however, gained records for all intermediate distances. Of course, these records were not necessarily achieved by competitiors of the same physiological capacity.

It must be emphasized that the results given here were obtained in an attempt to find, physiologically, the optimum method of employing human muscular effort for conversion to other forms of energy. For this purpose, where stationary cycling has to be considered, the position at about 63° with back-rest has many advantages, though it is doubtful whether these hold for a moving machine where the conventional design is mechanically much simpler.

SUMMARY

1. Results are given which show the effect on the efficiency of bicycle pedalling when the bicycle seat, with an added back-rest, is moved into different positions round the arc of a circle whose centre is the pedal axle.

2. It is found that there are two alternative optimum positions: one, the normal position adopted for the saddle in modern bicycles for which a line joining the rear of the seat to the pedal axle makes an angle of about 26° to the vertical; the other, with a back-rest supporting the pelvis, for which the angle quoted is approximately 63° ($\pm$ 10°). No significant difference between the efficiency of pedalling in these two positions was detected.

3. 'Step-up' experiments on two subjects are given, showing the resulting effects on various physiological functions as the work-loads were increased till the subject was exhausted. They are related to similar previous work.

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THE EFFECTS OF CHANGES IN DIETARY PROTEIN ON THE COMPOSITION AND STRUCTURE OF THE LIVER CELL

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It has been known for a considerable time that the protein content of the liver is dependent on the quantity of the dietary protein intake. Addis, Poo & Lew (1936 *a, b*) were the first to demonstrate that, on fasting, the liver lost protein more rapidly than any other tissue of the body. After a 2 days' fast the liver lost 20 % of its original protein content, while the losses from the heart, kidney and other organs amounted to only 4 % of their original protein contents. Further, the same authors, in a later paper (Addis *et al.* 1936 *c*), found that the liver, during the first 2 days on a protein-free diet lost more protein than any other organ, while the rate of the loss from the second to the tenth days was not very different for the various organs examined. On giving a 74 % casein diet to rats which had been on a protein-free diet, signs of increase in the protein content of the liver became noticeable after 12 hr. and were definite after 17 hr.

It appeared to be of interest to study in detail the changes which take place in the liver when the protein content of the diet is changed. The physiological significance of the phenomenon described by Addis and his collaborators must, to a great extent, depend on the manner in which the 'labile' protein is incorporated in the liver cell; viz. whether it is deposited as inert storage material or integrated into the cytoplasm of the cell, thus becoming part of living matter. It was intended to examine also if there is a correlation between these chemical changes and the cytological changes which have repeatedly been observed in the liver cell after alterations in the dietary protein intake (Berg, 1914, 1922 *a, b*; Noël, 1923; Elman & Heifetz, 1941; Elman, Smith & Sachar, 1943).

The literature bearing on this subject has been reviewed recently (Kosterlitz & Campbell, 1945–6), so that it may be permissible not to refer to it in detail. Some of the points made in this paper have already been communicated in brief notes (Kosterlitz & Cramb, 1943; Kosterlitz, 1944 *a, b*).

METHODS

Animals

Rats of the hooded strain of the Rowett Research Institute were used. If not stated otherwise, female animals, 15–18 weeks old, were selected. The animals were bred in this laboratory and kept at a temperature of $70 \pm 5°$ F. They were fed on a stock diet differing from that described by Thomson (1936) in its higher content of dried skim milk, viz. 14 instead of 7 %. The nitrogen content of this diet was 3·16 %. Except for a daily supply of greens, and of milk during pregnancy and lactation, no other food was given. The weights of the animals were recorded weekly, and animals not growing at a normal rate were excluded from the experiments.

Preparation of the diets

In the earlier experiments the basal diet (type 1) consisted of 2 % agar, 3 % salts No. 351 (Hubbell, Mendel & Wakeman, 1937), 8 % dried brewers' yeast, 76 % sucrose and 11 % lard. The lard was supplemented with vitamins A, D and E by adding 1·05 % (v/w) of a mixture 1 in 15 of α-tocopheryl acetate in radiostoleum (B.D.H.). When protein (casein or gelatin) was incorporated in the diet, it replaced the sucrose of the diet. To diets containing no casein, 0·4 % disodium hydrogen phosphate and 0·3 % potassium dihydrogen phosphate were added.

In the majority of experiments the basal diet of type 2 was used. This consisted of 2 % agar, 3 % salts No. 351, 25 % sucrose, 60 % potato starch, and 10 % lard. Vitamins A, D and E were added in the same manner as in the diet of type 1. A daily supplement (0·5 ml.) was given which contained 30 μg. aneurin, 30 μg. pyridoxin, 50 μg. riboflavin, 100 μg. Ca-d-pantothenate, 100 μg. nicotinic acid, 1 mg. inositol, and 17·3 mg. choline hydrochloride. In addition, the rats received daily whole-liver concentrate (Glaxo Laboratories, Ltd.) corresponding to 2 g. fresh liver. In diets containing less than 18 % casein, a mixture of four parts of disodium hydrogen phosphate and three parts of potassium dihydrogen phosphate was given to replace the P of the casein.

Care of animals during experimental period

During the experimental period, which usually lasted for 1 week, the rats were placed in individual cages consisting of $\frac{1}{2}$ in. mesh to prevent coprophagy. The food was supplied in heavy china conical dishes; access to the food was possible only through a circular opening at the upper surface. Except in the case of unpalatable food, there was very little scattering of food. The cages were inspected every morning and spilt food returned to the dishes. The animals were weighed and fed between 4 and 5 p.m. The vitamin B supplements were given in a separate small dish before the food was placed in the cages, and usually they were readily taken. If the supplements were not consumed within 30 min. they were added to the solid food. Water was given *ad libitum*.

Sampling of tissues for analysis

The rats were killed by decapitation between 2 and 3 p.m. and bled. The livers were then removed rapidly, washed in cold water and dried between filter paper. In order to ensure samples representative of the whole liver, small portions were taken from all lobes and pooled for analysis. The samples of tissue required for glycogen estimation were removed first.

Analytical methods

Estimation of glycogen. The method described by Kosterlitz & Ritchie (1943) was used.

Estimation of water content. The tissue was dried at $105°$ C. for 18 hr.

Estimation of total fatty acids plus unsaponifiable matter. These were estimated by a modification of Kumagawa & Suto's method (1908). The tissue (2·5–4·5 g.) to which 15 ml. 10 N-NaOH had been added, was digested on the steam bath for 1 hr. The mixture was allowed to cool, and after addition of 15 ml. 20 % (v/v) ethanol, it was heated on the hot-plate for 1 hr. After cooling and acidification with 13 ml. 20 N-H_2SO_4, the fatty acids were extracted with 25, 15, 15 and 15 ml. ether. The combined ether extracts were evaporated and the residue dried in vacuo over $CaCl_2$ for 1 hr. Then 15 ml. petroleum ether (b.p. 40–60° C.) were added, the solid broken up, and after 1 hr., the

extract was warmed to boiling-point and filtered through a fat-free paper into a weighed beaker. The extraction with petroleum ether was repeated twice and the extracts evaporated. After drying in vacuo over $CaCl_2$ for at least 1 hr., the residue was weighed. This value comprises total fatty acids and unsaponifiable matter.

Estimation of protein, phospholipin and nucleic acid. The liver sample was first extracted with trichloroacetic acid in order to remove water and acid-soluble nitrogen and phosphorus compounds. The lipoid P was estimated in the combined ethanol-ether extracts of the residue, and the nucleic acid P (residual P) and protein + nucleic acid N (residual N) in the residue after alcohol-ether extraction. Estimation of lipoid P after acid extraction was used by Teorell & Norberg (1932) and by Flock, Bollman & Mann (1936), while Euler & Schmidt (1934) have estimated nucleic acid P in tissue after trichloroacetic acid extraction followed by ethanol-ether extraction.

2 g. liver tissue were thoroughly ground in a glass mortar with 10 ml. 5 % trichloroacetic acid; after 5–10 min. the mixture was transferred to a centrifuge tube, the mortar washed twice with 5 ml. trichloroacetic acid, and the combined extract and washings centrifuged. The supernatant fluid was discarded. The residue was twice re-extracted with 10 ml. 5 % trichloroacetic acid. For the extraction of the phospholipin P the residue was treated twice with 20 ml. cold ethanol. This was followed by two extractions with 20 ml. hot ethanol-ether mixture (3 : 1), the liver residues and the ethanol-ether mixture in the centrifuge tubes being heated for 1 min. in a boiling water-bath. Finally, they were extracted with 20 ml. warm ether. The combined extracts were made up to 100 ml. with ethanol and P was estimated in 3 ml. samples. The extracted residue was dried in vacuo over $CaCl_2$ and then incubated with 20 ml. 0·25 N-NaOH at 37° C. for 24 hr. during which time all material was dissolved (apart from traces of fibrous material). Residual N was estimated in 0·08 ml. and residual or nucleic acid P in 1 ml. The extractions of acid-soluble and lipoid P were found to be quantitative.

P was estimated by Fiske & Subbarrow's method (1925), a Spekker photoelectric absorptiometer being used for the determination of colour intensity. N was estimated by Ma & Zuazaga's (1942) modification of the micro-Kjeldahl method with the following alterations: the time for digestion was prolonged to 30 min. after the reaction mixture had become clear, and for the distillation of NH_3 Markham's (1942) steam-jacketed apparatus was used.

Calculation of results

When the results are given for 100 g. of body weight, they generally refer to the body weight at the beginning of the feeding period (initial body weight). The final body weight is at least partly conditioned by changes in the lipid content of the body during the feeding period and therefore less uniform than the initial body weight.

Factors. The following were used for calculation: protein = protein N × 6·25; protein N = residual N − 1·7 × residual P. Phospholipin = lipoid P × 22·7 (Artom & Fishman, 1943); nucleic acid = residual P × 10·3. Neutral lipids = 1·1 × (total fatty acids − 15·3 × lipoid P). The values given for total fatty acids and for neutral lipids include unsaponifiable matter.

In order to estimate the relative concentrations of protein, phospholipin and nucleic acid, the following ratios were calculated: (1) protein × 100/protein + phospholipin + nucleic acid (= *protein index*), (2) phospholipin × 100/protein + phospholipin + nucleic acid (= *phospholipin index*), and (3) nucleic acid × 100/protein + phospholipin + nucleic acid (= *nucleic acid index*).

Histological technique

Since most of the phospholipin and ribonucleic acid of the liver is found in the basophil granules and in the chromophil ground substance of the cytoplasm, the main interest was focused on demonstrating a possible relationship between these structures and the results of the chemical analyses. Both basophil granules and chromophil ground substance can be readily recognized in sections stained with haemalum-eosin. The basophil granules were also stained by Schönholzer's (1936) modification of the Unna-Pappenheim method. Mitochondria were stained by Deane's modification (1942) of Regaud's technique. The number of nuclei was determined by the method described by Brues, Drury & Brues (1936).

The blood content of the liver

Without perfusing the liver with Ringer-Locke's fluid, it is impossible to remove the blood completely. Fenn (1939), by counting red blood cells, estimated that the livers of rats killed by stunning and then bled from the carotid arteries, contained between 0·7 and 3 % of blood and, if the animals were not bled, between 3·7 and 5·2 % of blood. These results have, on the whole, been confirmed in this series of experiments by estimating the blood contents spectrophotometrically in saline extracts of the liver (Dr T. N. Morgan). In twelve animals which had been bled well, the mean blood content of the livers was 1·86 ml./100 g. liver with a standard deviation of 0·54 ml./100 g. liver. The protein content of rat blood is approximately of the same magnitude as that of liver, the lipoid P content amounts to about 6–8 % of that of liver while the nucleic acid P content is negligible. If these values are taken into account, an admixture of about 2 % of blood will cause an error of about 2 % in the estimation of the protein content of the liver, expressed as mg./100 g. liver, while the estimations of phospholipin and nucleic acid are not significantly affected. In view of the smallness of the error no attempt was made to remove the blood from the liver by perfusion.

RESULTS

Changes in the chemical composition of the livers

First series of experiments

This series was designed to give information on changes in the composition of liver tissue when rats were fed on diets which, with regard to their protein content, were either normal, excessive or deficient in quantity and quality. The basal diet was of type 1, with the exception of the 60 % casein diet where it was of type 2. The effect of fasting after the stock, normal or low protein diets was also studied.

From the results summarized in Table 1 a, it follows that, in comparison with results from rats on the stock diet, the weight of the liver in terms of 100 g. body weight was slightly raised in rats on the high protein diet (60 % casein), not significantly changed on the diet containing adequate quantities of protein (20 % casein + 8 % yeast), slightly lowered on the protein-deficient diets (18 % gelatin + 8 % yeast, 8 % yeast), and considerably reduced in animals fasted for 24 or 48 hr. The percentage water content of the livers showed no significant difference between the individual groups of rats. The protein concentration of the livers of the animals on the 20 % casein + 8 % yeast diet was slightly lower than that of the stock rats, and greatly reduced in the rats on the protein-deficient diets. In the fasted rats, the protein concentration was either normal or raised. The phospholipin concentration was slightly lowered on the diets with a high or normal protein content and showed a marked decrease in the livers of rats fed on protein-deficient diets. In the fasted animals the phospholipin concentration was scarcely different from that found in the stock rats. The nucleic acid concentration was unchanged in fed animals except those on a low-protein diet, where it was low. In fasted rats it was, however, noticeably raised. The glycogen content was raised in all animals fed on the experimental diets; the rise was particularly evident in

TABLE 1. Composition of livers of rats fed on different diets

(a) Results expressed in g./100 g. liver

Diet	No. of rats	Initial weight of rats (g.)	Weight of liver (g./100 g. body wt.)	Water	Protein	Phospholipin	Nucleic acid	Glycogen	Neutral lipids
							(g./100 g. liver)		
Stock	7	226 ± 5	3·56±0·11	70·3 ±0·20	17·3 ±0·18	3·37±0·03	1·31±0·021	2·27±0·23	1·49±0·08
60 % casein	4	222 ± 5	3·96±0·15	69·75±0·16	17·8 ±0·11	3·05±0·045	1·30±0·006	2·98±0·27	1·67±0·07
20 % casein + 8 % yeast	6	213 ± 6	3·72±0·08	70·1 ±0·20	16·6 ±0·13	2·99±0·03	1·30±0·007	3·29±0·41	1·85±0·10
18 % gelatin + 8 % yeast	6	221 ± 6	3·27±0·12	69·8 ±0·26	15·1 ±0·38	2·86±0·06	1·32±0·039	3·03±0·53	4·18±0·26
8 % yeast	6	222 ± 8	3·24±0·08	71·05±0·46	13·85±0·29	2·50±0·045	1·24±0·021	5·71±0·22	2·83±0·39
24 hr. fast:									
After stock	5	225 ± 9	2·91±0·07	70·9 ±0·16	17·7 ±0·16	3·51±0·08	1·43±0·020	0·11±0·01	1·75±0·08
After 20 % casein + 8 % yeast	6	226 ± 4	2·80±0·08	70·1 ±0·32	18·1 ±0·07	3·32±0·05	1·51±0·020	0·11±0·01	2·97±0·18
After 8 % yeast	6	223 ± 4	2·78±0·07	71·7 ±0·22	16·9 ±0·20	3·16±0·06	1·55±0·024	0·16±0·02	2·87±0·26
48 hr. fast:									
After stock diet	6	232 ±10	2·51±0·06	70·35±0·21	18·3 ±0·18	3·55±0·05	1·53±0·017	0·47±0·16	1·75±0·20

(b) Results expressed in mg./100 g. body weight

Diet	Protein	Phospholipin	Nucleic acid	Protein + phospholipin + nucleic acid
Stock	613±11·8	120±3·4	46·5±0·97	780±15·8
60 % casein	706±22·1	120±2·7	51·5±1·55	878±26·1
20 % casein + 8 % yeast	618±14·5	111±9·0	48 ±0·30	777±18·3
18 % gelatin + 8 % yeast	491±10·7	93±2·4	43 ±0·97	627±13·6
8 % yeast	449± 5·9	81±1·9	40·5±0·79	570± 8·4
24 hr. fast:				
After stock	513±14·3	102±4·3	41·5±1·17	657±19·5
After 20 % casein + 8 % yeast	507±15·7	93±2·0	42·5±1·58	642±18·8
After 8 % yeast	471±14·5	88±3·1	43 ±1·43	602±18·8
48 hr. fast:				
After stock	459±12·1	89±1·2	38·5±0·92	586±13·9

(c) Indices

	Protein index	Phospholipin index	Nucleic acid index
Stock	78·6±0·19	15·4±0·18	6·0 ±0·05
60 % casein	80·5±0·29	13·7±0·18	5·85±0·01
20 % casein + 8 % yeast	79·5±0·14	14·3±0·13	6·2 ±0·06
18 % gelatin + 8 % yeast	78·3±0·18	14·9±0·18	6·85±0·07
8 % yeast	78·8±0·18	14·2±0·15	7·05±0·06
24 hr. fast:			
After stock	78·2±0·17	15·5±0·24	6·35±0·06
After 20 % casein + 8 % yeast	78·9±0·25	14·5±0·24	6·6 ±0·07
After 8 % yeast	78·2±0·14	14·6±0·18	7·15±0·10
48 hr. fast:			
After stock	78·2±0·25	15·2±0·20	6·55±0·07

the animals on the low-protein diet. The fasted animals had the expected low glycogen contents. The concentration of neutral lipids was raised in rats on the protein-deficient diets and in the rats fasted for 24 hr. after diets adequate or inadequate in protein. These results suggest that, whenever there was a deficiency in dietary protein, the livers lost protein, phospholipin and, to a less extent, nucleic acid. In the animals fed on protein-deficient diets, there was a decrease in the concentrations of these three constituents with a concomitant increase in glycogen or neutral lipid or both. There was only little loss in liver weight. On the other hand, in the fasted animals there was a slight increase in the concentrations of protein, phospholipin and nucleic acid together with a very pronounced loss in glycogen and liver weight.

When the results were calculated per 100 g. initial body weight instead of per 100 g. of liver weight, the variations in liver size caused by changes in the glycogen and neutral lipid contents were eliminated (Table 1 b). If the stock diet be again taken as the standard for comparison, the livers of rats on the high-protein (60 % casein) diet showed significant increases in the protein and nucleic acid contents while the phospholipin content remained unchanged. No significant changes were observed in the animals on the 20 % casein + 8 % yeast diet. The rats fed on the protein-deficient diets, particularly those on the 8 % yeast diet, showed marked decreases in all three liver constituents, the losses of nucleic acid being smaller than those of protein or phospholipin. Considerable losses of all three substances were incurred after a fast lasting 24 or 48 hr., and these were relatively greater during the first 24 hr. period than during the second 24 hr. period. A 24 hr. fast following a low-protein diet caused no further decrease in the protein, phospholipin and nucleic acid contents of the liver.

Calculation of the protein, phospholipin and nucleic acid indices (see p. 196) showed that the protein and phospholipin indices were remarkably constant, with small fluctuations around 78·5 for protein and 14·8 for phospholipin (Table 1 c). On the high-protein diet only there was an increase of the protein index together with a fall of the phospholipin index. The nucleic acid index, on the other hand, showed a definite tendency to rise whenever there was a loss in protein, phospholipin and nucleic acid, this being particularly pronounced on the rats receiving the low-protein diets. As shown later the relative increase in nucleic acid is best explained by assuming that only cytoplasmic or ribonucleic acid is lost and not nuclear or deoxyribonucleic acid.

This view is supported by the absence of any significant decrease in the number of liver nuclei, calculated per 100 g. of body weight, in rats on protein-deficient diets or in fasted rats (Table 2). Although the count in the fasted rats seemed to indicate a rise in the number of nuclei, this was statistically not significant, except in the case of the rats fasted after the 8 % yeast diet. It should be noted, however, that the experimental error of the method is

high, as can be seen from the relatively large standard errors of the means. The size of the liver cells may be estimated as the reciprocal of the number of cells per 100 g. liver. There was very little change in size whether the animals were fed on a normal or a protein-deficient diet. On the high-protein diet, there was an increase in the cell size, and in the fasted rats a very marked decrease. That the deposition of glycogen compensates for the loss in protein, phospholipin and nucleic acid from the livers of rats fed on low-protein diets and thus prevents a decrease in the cell size, can be very clearly demonstrated by calculating the analytical results per 1000 liver cells (Kosterlitz, 1946).

TABLE 2. Number of cells in the livers of rats fed on different diets

Diet	Nuclei × 10⁶/100 g. body weight	Binucleated cells/1000 cells	Cells × 10⁸/100 g. liver
Stock	609 ± 32	108 ± 4	152 ± 2
60 % casein	550 ± 33	116 ± 6	123 ± 5
20 % casein + 8 % yeast	583 ± 28	89 ± 5	143 ± 7
18 % gelatin + 8 % yeast	543 ± 21	103 ± 7	150 ± 10
8 % yeast	553 ± 42	95 ± 5	153 ± 9
24 hr. fast:			
After stock	675 ± 40	128 ± 12	203 ± 11
After 20 % casein + 8 % yeast	650 ± 29	117 ± 4	206 ± 14
After 8 % yeast	657 ± 16	102 ± 5	213 ± 4
48 hr. fast:			
After stock	600 ± 27	122 ± 2	210 ± 10

Second series of experiments

In this series, rats were fed on a protein-free diet for varying periods in order to study the rates at which protein, phospholipin and nucleic acid were lost from the liver.

Groups of four rats were transferred from the stock diet to the basal protein-'free' diet of type 2, for periods ranging from 1 to 28 days (Table 3 a). The daily N intake was approximately 7·5 mg., 5 mg. being derived from the whole-liver concentrate. The rats lost weight at a rate of about 12 g. per week. The liver weight decreased during the first 24 hr. on the protein-free diet but after that remained remarkably constant. The concentration of water in the liver remained constant for the first fortnight and fell slightly during the second fortnight due to the increase in the neutral lipid concentration. This latter was raised during the first 2 days of the protein-free diet, fell to normal on the fourth day and then showed a continuous rise which became rather steep during the second fortnight of the experiment. Apparently, in the absence of dietary protein, the dosage of the lipotropic factors, choline and inositol, was not quite sufficient to prevent deposition of excessive quantities of neutral lipids. The protein and phospholipin concentrations fell very markedly during the experiment, while the fall in nucleic acid was considerably smaller. There was a significant increase in the glycogen concentration of the liver from the second day onwards. The loss in protein, phospholipin and nucleic acid was well

TABLE 3a. Composition of livers of rats fed on a protein-free diet for varying periods

Diet	No. of rats	Initial weight of rats (g.)	Loss of weight during experiment (g.)	Weight of liver (g./100 g. initial body wt.)	Water	Protein	Phospholipin	Nucleic acid (g./100 g. liver)	Glycogen	Neutral lipids
Stock diet	7	226±5	—	3·56±0·11	70·3 ±0·20	17·3 ±0·18	3·37±0·03	1·31±0·021	2·27±0·23	1·49±0·08
22 hr. protein-free diet	4	217±5	5	3·10±0·06	70·4 ±0·32	16·6±0·14	3·13±0·06	1·31±0·030	1·87±0·39	3·03±0·16
46 hr. protein-free diet	4	214±7	9	3·07±0·06	71·0 ±0·41	15·2±0·19	2·86±0·03	1·26±0·025	3·91±0·47	2·37±0·31
4 days protein-free diet	4	222±10	13	3·16±0·06	71·35±0·53	14·2±0·25	2·69±0·05	1·20±0·016	5·41±0·41	1·83±0·15
7 days protein-free diet	4	222±4	11	3·27±0·08	70·7 ±0·16	13·4±0·11	2·61±0·10	1·18±0·050	6·70±0·79	2·63±0·18
14 days protein-free diet	4	213±11	28	3·10±0·05	70·65±0·71	13·2±0·11	2·43±0·03	1·26±0·035	5·34±0·44	4·21±0·56
21 days protein-free diet	4	223±8	35	2·77±0·23	69·05±0·94	12·5±0·42	2·33±0·07	1·22±0·045	5·67±0·32	6·80±1·13
28 days protein-free diet	4	219±9	43	3·07±0·10	67·25±1·28	11·8±0·34	2·32±0·10	1·22±0·041	5·42±0·66	9·87±1·54
7 days protein-free diet with 40 % lard	4	224±3	13	3·03±0·13	70·2 ±0·31	13·2±0·41	2·64±0·09	1·26±0·31	4·61±0·45	4·85±0·31

TABLE 3b. Protein, phospholipin and nucleic acid indices of liver of rats fed on a protein-free diet

Diet	Protein + phospholipin + nucleic acid (mg./100 g. initial body wt.)	Protein index	Phospholipin index	Nucleic acid index
Stock	780±16	78·6±0·19	15·4±0·18	6·0 ±0·05
22 hr. protein-free diet	654±17	78·9±0·17	14·8±0·14	6·25±0·05
46 hr. protein-free diet	594±12	78·7±0·18	14·8±0·15	6·5 ±0·08
4 days protein-free diet	572±11	78·5±0·27	14·9±0·26	6·65±0·05
7 days protein-free diet	561±7	77·9±0·11	15·2±0·16	6·85±0·12
14 days protein-free diet	522±9	78·1±0·22	14·4±0·12	7·5 ±0·14
21 days protein-free diet	443±21	77·9±0·21	14·5±0·17	7·6 ±0·07
28 days protein-free diet	471±10	77·0±0·18	15·1±0·35	7·9 ±0·26
7 days protein-free diet with 40 % lard	618±19	77·1±0·24	15·5±0·21	7·35±0·04

balanced by the increase in glycogen and neutral lipids so that the livers lost
no weight during the course of the experiment. This is borne out when the
values are calculated per 100 g. of initial body weight (Text-fig. 1). It was then
found that liver protein and phospholipin fell rapidly during the first 4 days
of the protein-free regimen, while nucleic acid showed a much smaller decrease.
After the fourth day all three substances were lost more slowly. The glycogen
increased during the first week, while the neutral lipid changed only little.
After that, the glycogen decreased slightly while the neutral lipid started to
rise. The loss of protein + phospholipin + nucleic acid occurred at a rate similar
to that of protein. Campbell & Kosterlitz (1946a) have shown that the curve
obtained consists of an exponential and a linear component. The linear
component is probably a measure of endogenous N metabolism, while the
exponential component represents the rapid loss of protein which is peculiar
to the liver during the first few days of a protein-free regimen. As far as the
protein, phospholipin and nucleic acid indices are concerned (Table 3b), a small
decrease in the protein index and no definite changes in the phospholipin index
was observed. On the other hand, the nucleic acid index showed an increase
which became more and more marked the longer the animals were kept on
the protein-free diet.

Substitution of the carbohydrate moiety by fat led to a more rapid loss of
protein from the liver and to an accelerated rise of the nucleic acid index.
There was also an increased deposition of neutral lipid (Table 3).

Changes in the histological appearance of the liver

The changes in the protein, phospholipin and nucleic acid contents of liver
tissue can be correlated with changes in the histological appearance of the
liver cells. In this respect, the chromophil ground substance and the basophil
granules of the cytoplasm are of particular interest in that they account for
almost all the phospholipin and ribonucleic acid present in the cytoplasm
(Bensley, 1942; Brachet & Jeener, 1944; Claude, 1946).

Pl. 1, fig. 1 shows the appearance of a liver section from a rat fed on the
stock diet which may be considered adequate so far as quality and quantity
of the protein is concerned. The basophil granules and the chromophil ground
substance of the cytoplasm can be clearly recognized.

The liver cells of rats which had been fed on a protein-free diet for varying
periods usually but not always showed a slight diminution in size (Pl. 1,
figs. 2–5). The remarkable feature, however, was the rarefaction of the cyto-
plasm due to a pronounced loss of basophil granules and chromophil ground
substance, particularly in the peripheral parts of the lobules. The chemical
analyses of these livers (Tables 1, 3) indicated a decrease in the protein,
phospholipin and nucleic acid concentrations together with a rise in the levels
of glycogen and, in the later stages of the protein-free regimen, also of lipid.

mg./100 g. body weight
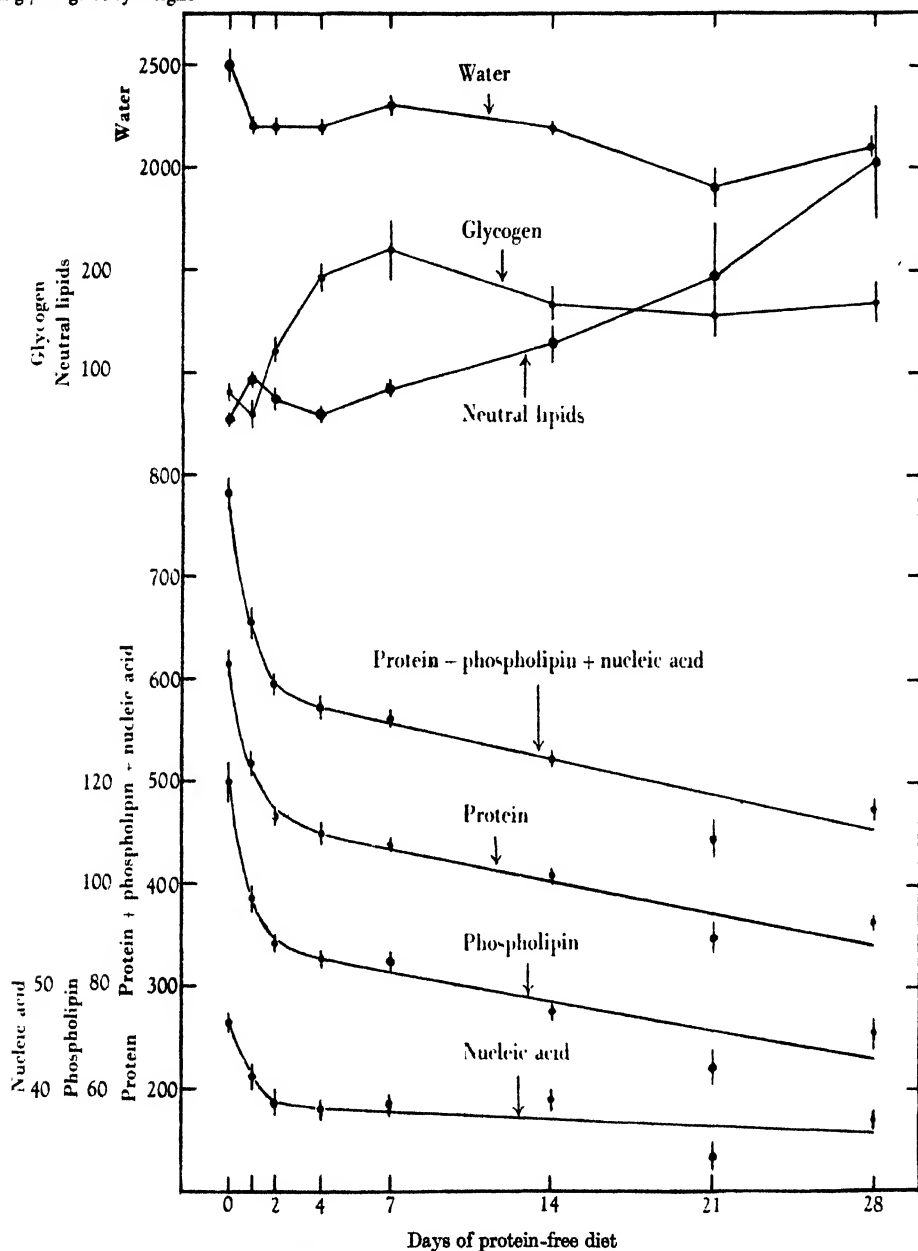
Text-fig. 1. Changes in composition of liver tissue in rats transferred from the stock diet to a protein-free diet. (The curves were fitted by eye.)

These findings confirm and extend the results of Elman *et al.* (1943) who correlated the histological appearance of the liver with its protein content. According to these authors, livers of dogs and rats fed on low-protein diets for 2–3 weeks had chemically a low-protein concentration and histologically a loss in stainable cytoplasm; the cells showed large clear spaces which were found to be filled with glycogen.

The loss of basophil granules and of chromophil ground substance was very rapid during the first 2 days of a protein-free diet (Pl. 1, figs. 2, 3), slowed down between 2 and 4 days (Pl. 1, fig. 4) and was small between 4 days and 4 weeks (Pl. 1, fig. 5). This is in agreement with the rate of loss of protein, phospholipin and nucleic acid from the liver and, as will be shown later, is caused by the disappearance of 'labile' liver cytoplasm.

The liver cells of rats fed for 1 week on a diet lacking in essential amino acids (gelatin) showed a decrease in the chromophil ground substance and the basophil granules similar to that found after 1 day of a protein-free diet (Pl. 1, fig. 7). This again agrees fairly well with the results of the protein, phospholipin and nucleic acid estimations.

Fasting for 48 hr., after the stock diet, resulted in small, more or less homogeneous, liver cells staining more intensely than the liver cells of fed rats (Pl. 1, fig. 6). There was, however, a decrease in basophil granules. It should be stressed that, although fasting for 48 hr. had the same effect on the *absolute amount* of protein, phospholipin and nucleic acid present in the liver as had feeding of a protein-free diet for 48 hr., the histological appearances of the livers were very different (Pl. 1, figs. 3, 6). The results of the chemical analyses indicate that these differences were due mainly to variations in the glycogen contents of the livers. Thus, the glycogen-poor livers of the fasted rats had small cells with high *concentrations* of protein, phospholipin and nucleic acid while the glycogen-rich livers of the rats fed on the protein-free diet had cells of only slightly diminished size with low *concentrations* of protein, phospholipin and nucleic acid.

When rats were fed on a low-protein diet (8 % yeast) for 1 week and then fasted for 24 hr., the rarefied appearance of the cytoplasm disappeared; the liver cells then resembled those of rats fasted after the stock diet. As shown by chemical analysis, fasting caused the glycogen to disappear but there was no further loss in the absolute amounts of protein, phospholipin and nucleic acid. In confirmation of Noël's (1923) observations, the livers of rats fed on diets deficient in protein showed a decrease in the number of cytoplasmic structures stainable by Regaud's technique. These comprise mitochondria and 'protéoblastes'.

DISCUSSION

The results presented in this paper fully confirm the findings of Addis *et al.* (1936*a–c*), viz. that the protein content of the liver is dependent on the dietary protein intake. The following possible interpretations of this phenomenon have to be considered. First, protein may accumulate in the liver as inert 'storage' or 'deposit' protein. Secondly, the changes in liver protein may be associated with changes in other essential cell constituents. In this case, the variations in the protein content of the liver may be caused either by variations in the amount of cytoplasm present in the individual liver cells, or by variations in the number of liver cells or, finally, by variations in both the number of cells and their cytoplasmic contents. The absence of statistically significant changes (with one exception) of the numbers of liver cell nuclei in fasted rats and in rats fed on protein-deficient diets makes it unlikely that variations in the number of liver cells are to any great extent responsible for the observed phenomenon.

If the decrease or increase in liver protein be due to a loss or gain of inert deposit protein, then a change should be expected in the ratios of protein to phospholipin and of protein to nucleic acid. However, the protein and phospholipin indices, which are a measure of the protein/phospholipin ratios, remain remarkably constant. This means that the changes in liver protein are accompanied by corresponding changes in liver phospholipins. The only exception to this rule has been found in rats on the high-protein diets, in which a slight increase was observed in the protein index together with a small fall in the phospholipin index.

As far as the protein-nucleic acid ratio is concerned, a complication is introduced by the fact that the values obtained for nucleic acid comprise both nuclear deoxyribonucleic and cytoplasmic ribonucleic acids. Although nucleic acid is lost from the liver when liver protein decreases, this loss proceeds at a slower rate than that of protein. Therefore a fall in the protein-nucleic acid ratio or a rise in the nucleic acid index is found in such experiments. It has already been suggested (Kosterlitz, 1944*a, b*) that this phenomenon may be due to the fact that cytoplasmic ribonucleic acid is lost from the liver but not nuclear deoxyribonucleic acid. This suggestion has been verified for the fasting rat by Davidson (1945*b*) who found that the ribonucleic acid content of the liver fell while its deoxyribonucleic acid content remained unaltered. There is so far only circumstantial evidence which makes it likely that these findings also hold for the rat fed on a protein-free diet. Analyses by various authors (Davidson & Waymouth, 1944*a*; Davidson, 1945*a, b*; Schmidt & Thannhauser, 1945; Schneider, 1945; Hammarsten & Hevesy, 1946) have shown that in rats' livers ribonucleic acid accounts for between 65 and 80 % of the total nucleic acid content, the remainder being deoxyribonucleic

acid. It can be shown that the nucleic acid indices observed in the second series of experiments (Table 3 *b*) are in fairly close agreement with indices calculated on the assumption that the total nucleic acid consisted of a mixture of 30–40 % deoxyribonucleic acid and 70–60 % ribonucleic acid.

It seems then that the changes in the protein content of the liver in various nutritional conditions are accompanied by similar changes in the phospholipin and the cytoplasmic ribonucleic acid contents. Recent investigations by Bensley (1942), Claude (1941, 1943*a, b*, 1946), Hoerr (1943) and Lazarow (1943) have shown that the cytoplasm of the liver cell is composed of particulate and interparticulate matter. The particulate matter consists of phospholipin-ribonucleoprotein complexes which appear to account for almost all the phospholipin and ribonucleic acid present in the cytoplasm of the liver cell (Bensley, 1942; Brachet & Jeener, 1944; Claude, 1946). The interparticulate continuous aqueous phase of the cytoplasm contains soluble proteins, mainly globulins, electrolytes and metabolites but practically no phospholipins and ribonucleic acid. Since the phospholipin concentration in whole liver tissue is considerably lower than that in the particulate matter and since the protein-phospholipin ratio does not undergo any considerable variation, it follows that the whole cytoplasm, particulate and interparticulate, takes part in the changes caused by fasting or by variation in the dietary protein. There is, therefore, no evidence in favour of an inert deposit protein in the liver, a conclusion which is supported by the fractionation experiments of Luck (1936), who could not single out any of the liver proteins as reserve, labile, or cell inclusion protein. The significance of these considerations is underlined by the fact that at least some of the enzymes of the liver cell are associated with the particulate matter of liver cytoplasm (Jeener & Brachet, 1941; Bensley, 1942; Lazarow, 1943; Brachet & Jeener, 1944; Chantrenne, 1944; Claude, 1946).

The time relationships of the losses of liver cytoplasm are of considerable interest. From the results shown in Table 3 *b*, it can be calculated that female rats, which were transferred from the stock diet to a protein-free diet, lost of their initial liver cytoplasm (protein + phospholipin + nucleic acid) 16·2 % in 1 day, 23·8 % in 2 days, 26·7 % in 4 days, and 28·1, 33·1 and 39·6 % in 1, 2 and 4 weeks respectively. If female rats fed previously on the stock diet were fasted for 1 day, they lost 15·8 % and, if fasted for 2 days, 24·9 % of their initial liver cytoplasm. Thus, the effect of fasting on liver cytoplasm is similar to that of feeding a protein-free diet for a similar period. As the loss of cytoplasm from the liver in the first few days of a protein-free regimen is so much more rapid than that observed from about the fourth day onwards, it has been proposed to call the easily lost fraction 'labile' liver cytoplasm (Kosterlitz, 1944*b*). 'Labile' liver cytoplasm appears to be present whenever the protein intake is above requirement and it varies directly with the logarithm of the protein intake (Kosterlitz & Campbell, 1946). While the loss of 'non-labile'

liver cytoplasm on a prolonged protein-free diet appears to be due to 'wear and tear' which is not replaced by dietary protein, the changes in 'labile' cytoplasm are probably the main source of those fluctuations of urinary N which made Rubner (1911) assume the presence of an unorganized 'storage protein'. It has been found (Campbell & Kosterlitz, 1946b) that rather more than half of the extra urinary N excreted during the first few days of a protein-free regimen can be accounted for by the N derived from the breakdown of 'labile' liver cytoplasm. Since analyses of the amino-acid patterns of the proteins or of the composition of the phospholipins of the 'labile' and 'non-labile' cytoplasms are not yet available, it cannot be stated whether or not they are identical in their chemical composition.

Elman & Heifetz (1941) and Elman *et al.* (1943) were probably the first authors to correlate cytological with chemical changes in livers of dogs and rats fasted or fed on normal or protein-free diets. They studied the effects of dietary changes lasting for 2–3 weeks. The term hydropic degeneration was applied to the histological picture of the liver of an animal which had been fed on a protein-free, high carbohydrate diet. On the other hand, the histological appearance of a liver of a fasted animal was spoken of as atrophy. In the experiments described in the present paper, it has been found that qualitatively, if not quantitatively, similar histological changes are observed after such short periods as 2 days of protein deficiency, or fasting, respectively. Further, a fast of 24 hr. will convert 'hydropic degeneration' into 'atrophy', a change which does not entail any further loss of cytoplasm. Since the changes are so readily produced and are also reversible, it would appear to be advisable, at least for short-term experiments, to avoid the terms of hydropic degeneration and atrophy and to use in preference the non-committal terms of 'glycogen-rich and glycogen-poor hypocytoplasmic livers'.

Both the chromophil ground substance and the basophil granules of the liver cell appear to be of a particulate nature and to consist of phospholipin-nucleoprotein complexes. Claude (1943a, b, 1946) found that the chromophil ground substance corresponds to the submicroscopic 'microsomes'. Davidson & Waymouth (1946) suggested that the basophil granules may be identical with the 'large particles' (Bensley, 1942; Lazarow, 1943; Claude, 1943a, b, 1946). That there is a decrease of basophil granules in protein-deficiency was first observed by Berg (1914, 1922a, b) who held that they represented storage protein. Brachet (1941), Brachet & Jeener (1944), however, pointed out that the staining properties of these granules are due to their content of ribonucleic acid and not of protein, since after incubation with ribonuclease, the granules no longer stained with pyronin. The same observation was independently made by Biesele (1944) and Davidson & Waymouth (1944b, 1946). These facts together with the chemical findings of Luck (1936) and those reported in this paper give no support to Berg's hypothesis of 'cell inclusion proteins'.

Little is known about the physiological significance of labile liver cytoplasm. It is unlikely that its main function is that of storage of protein, phospholipin and ribonucleic acid, since the quantities involved are relatively small. Thus, the N of 'labile' liver cytoplasm could not account for the large quantities of urinary N excreted after injuries. It is also improbable that 'labile' liver cytoplasm is identical with the 'mobile or dispensable' cell proteins which are of importance in regeneration of plasma proteins (Madden & Whipple, 1940; Whipple & Madden, 1944) since this process still takes place in fasted animals. On the other hand, close relationships between 'labile' liver cytoplasm and protein metabolism have already been established (Kosterlitz & Campbell, 1946; Campbell & Kosterlitz, 1946a, b). The quantities of 'labile' cytoplasm present in the liver vary directly with the logarithms of the daily protein intake, the regression coefficients being greater for male than for female rats. Since ribonucleic acid is assumed to play an important role in protein synthesis (Caspersson, 1941) losses of 'labile' cytoplasm may possibly decrease the efficiency of the liver in this respect. Lastly, it is possible that the changes in the phospholipin contents of the liver may affect fat metabolism since the turnover of phospholipins is accelerated by lipotropic factors (Chaikoff, 1942).

SUMMARY

1. In livers of rats fed on diets adequate or deficient in protein the following substances were estimated: water, protein, phospholipin, nucleic acid, glycogen and neutral lipids. The chemical findings were correlated with cytological changes in the chromophil ground substance and the basophil granules of the cytoplasm.

2. Compared with rats fed on an adequate stock diet, fasted rats and rats fed on diets qualitatively and quantitatively deficient in protein lost from their livers considerable amounts of protein, phospholipin and nucleic acid. Rats fed on high-protein diets showed an increase in these three liver constituents. There was generally no change in the number of liver cell nuclei. Protein and phospholipin were lost or gained at practically identical rates while the changes in nucleic acid were of smaller magnitude. Indirect evidence is adduced which suggests that only cytoplasmic ribonucleic acid and not nuclear deoxyribonucleic acid is affected.

3. From these findings it is concluded that the changes in liver protein which are associated with variations in dietary protein intake, are not caused by gains or losses of an inert storage protein but by gains or losses of whole cytoplasm, both particulate and interparticulate matter being affected.

4. When rats were transferred from the stock diet to a protein-free diet, they lost of their liver cytoplasm 16·2 % in 1 day, 23·8 % in 2 days, 26·7 % in 4 days, and 28·1, 33·1 and 39·6 % in 1, 2 and 4 weeks respectively. Fasting for 1 or 2 days had similar effects. It is proposed to call the easily lost fraction

'labile' liver cytoplasm.' It cannot be stated whether there are any chemical differences between 'labile' and 'non-labile' liver cytoplasm.

5. The histological appearance of a liver which has lost its 'labile' cytoplasm depends on its content of glycogen and neutral lipids. The liver of the fasted rat has small but rather intensely staining cells and may be called 'glycogen-poor hypocytoplasmic'; on the other hand, the liver of the rat fed on a protein-free diet has poorly staining cells of approximately normal or slightly diminished size and may be called 'glycogen-rich hypocytoplasmic'. In both types of liver there appears to be an absolute diminution in the chromophil ground substance and the basophil granules of the cytoplasm.

6. The physiological implications of the changes in liver cytoplasm are discussed.

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EXPLANATION OF PLATE 1

Paraffin sections of livers fixed in neutral 5 % formaldehyde solution in 0·9 % NaCl and stained with haemalum-eosin. The prints have been over-exposed in order to emphasize cytoplasmic details. Magnification × 376.

Fig. 1. Liver from a rat fed on the stock diet.

Figs. 2–5. Livers from rats fed on a protein-free diet for 1, 2, 4 and 28 days respectively.

Fig. 6. Liver from a rat fed on the stock diet and fasted for 48 hr.

Fig. 7. Liver from a rat fed on the 18 % gelatin + 8 % yeast diet for 7 days. .
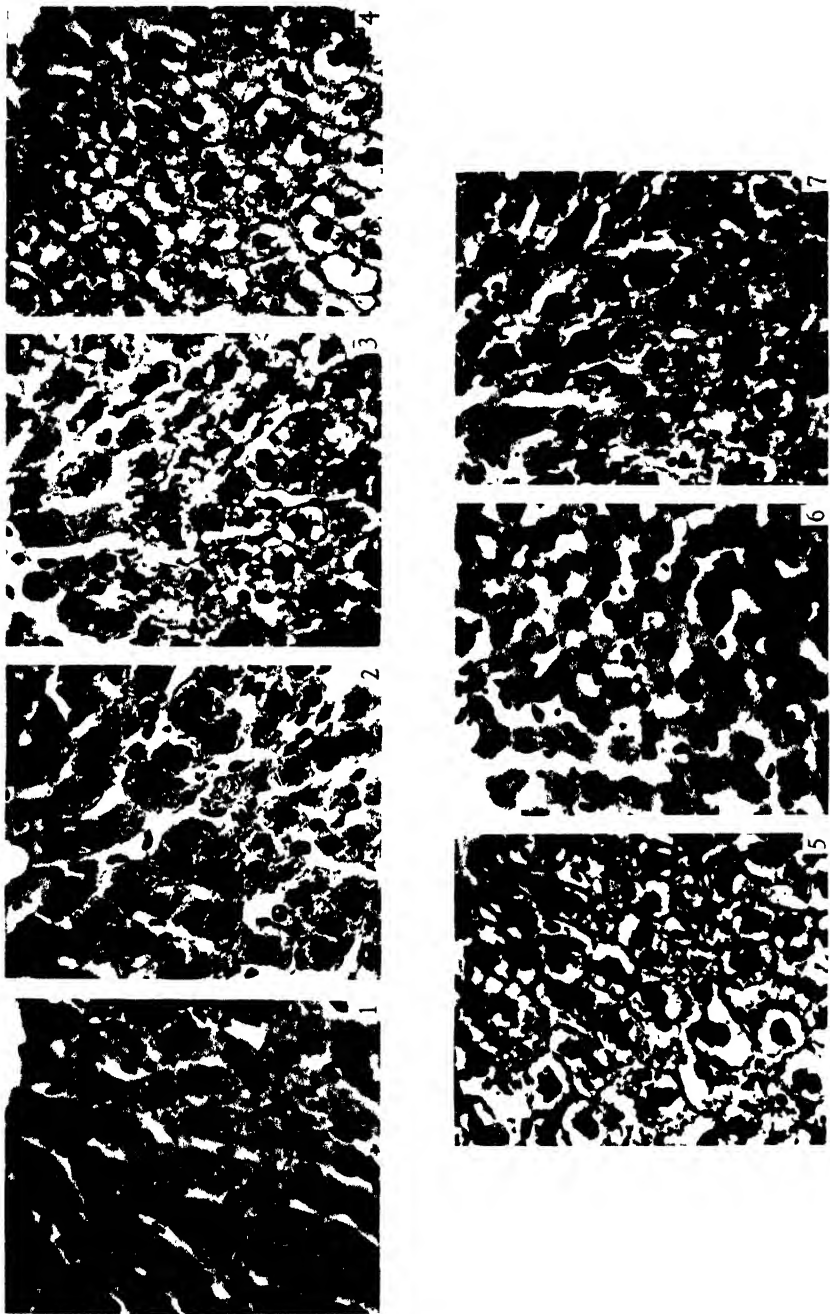
Figs. 1-7.

EFFECTS OF INTRATHECAL INJECTION OF KCl AND OTHER SOLUTIONS IN CATS. EXCITATORY ACTION OF K IONS ON POSTERIOR NERVE ROOT FIBRES

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In the course of experiments in which solutions of eserine were injected intrathecally in cats (Calma & Wright, 1947) we noted that rapid intrathecal injections of large volumes (0·5–2·0 c.c.) of physiological saline (0·9 % NaCl) produced changes in the circulation and modified skeletal muscle reflexes. We have extended these observations and have also examined in detail the effects of injection into the lower spinal theca of solutions of potassium salts (KCl or mixtures of $KH_2PO_4 + K_2HPO_4$), hypertonic solutions of NaCl, glucose and urea, and isotonic solutions of varying pH, on the diameter of the pupil, circulation, respiration and skeletal muscle reflexes. We have found that these solutions produce their effects, in the main, reflexly. In particular we have demonstrated that potassium salts specifically stimulate certain afferent fibres in the posterior nerve roots.

METHODS

Cats anaesthetized with chloralose (0·08 g./kg.) were used. The techniques employed for intrathecal injection and for recording somatic reflexes are described in detail in the preceding paper (Calma & Wright, 1947). As there explained, the spinal cord was exposed between L6 and S2: the dura was then pierced and a large metal cannula was introduced into the subarachnoid space at the level of the first sacral vertebra. The cannula was kept in position by means of a ligature passed round the dura; the cannula and membranes were then fixed to the muscles of the back. When solutions were injected at higher levels of the spinal cord, an injection needle was introduced into the subarachnoid space at the appropriate level and kept in position by means of a clamp fixed on the adjacent vertebrae. Other special procedures employed are described in the body of this paper. Carotid blood pressure was always recorded and the diameter of the pupil carefully noted. Respiration records were taken when required.

RESULTS

Effects of intrathecal injection of isotonic NaCl *solutions*

We re-examined the effects of intrathecal injection of solutions of 0·9 % NaCl at pH 7·4, slowly or quickly, at room temperature or at 37° C.; the volume of solution used varied from 0·1 to 2·0 c.c. The results were little affected by

temperature difference in the range specified. When the volume employed did not exceed 0·5 c.c. and the injection was carried out slowly, there was generally no effect on pupil diameter, blood pressure, respiration or somatic spinal reflexes. Occasionally such volumes of isotonic saline produced a small rise or fall (Fig. 10) of blood pressure (i.e. less than 25 mm. Hg) without change in pupil diameter or somatic reflexes. When larger volumes (0·5–2·0 c.c.) of fluid were injected intrathecally they more commonly produced conspicuous changes, e.g. a rise of blood pressure of 50–70 mm. Hg and well-marked pupil dilatation. In these experiments there was usually evidence of increased fluid pressure in the subarachnoid space, suggesting that the response was the result of mechanical stimulation of nerve elements within the theca. Positive reactions were especially marked after repeated intrathecal injections at short intervals, or when the preparation employed had had the subarachnoid space blocked in the mid-thoracic region by a tight ligature tied round the dura and enclosed spinal cord. In some instances the tip of the cannula through which the intrathecal injections were made, had shifted and become embedded in the terminal part of the cauda equina which would consequently be mechanically acted on when the fluid was injected. In animals under very light chloralose anaesthesia gentle traction on the spinal membranes (e.g. by attaching a syringe containing 0·2–0·4 c.c. of saline to the cannula) may cause a considerable blood-pressure rise (e.g. 50 mm. Hg), although intrathecal injection of 0·5 c.c. of saline might be without effect. We have frequently confirmed that pulling on a cut posterior nerve root, or a gentle pinch with forceps, produces a reflex rise of blood pressure and pupil dilatation. In our experience mechanical stimulation of the exposed dorsal aspect of the spinal cord (with the dura opened and retracted), brushing or pouring saline on the cord was generally ineffective. In attempting to interpret the significance of these results the following considerations are relevant. O'Connell (1946) in discussing the clinical signs of meningeal irritation has drawn attention to the exquisite sensitivity of the posterior nerve roots to mechanical stimulation as seen·at operation in man and to the fact that characteristic root pain develops when 'one of them is stretched by the downward movement of an attached tumour before a wave of cerebrospinal fluid produced by coughing or jugular compression. Having gained entry to the spinal medulla...the sensitivity of the posterior nerve roots to mechanical stimuli is greatly diminished. Again at its peripheral end once a posterior nerve root has penetrated the dura it is provided with a fibrous tissue sheath and is less sensitive to stimulation.' It seems very probable that, in our experiments, tugging on the meninges or injection of fluid would stimulate the posterior nerve roots. The question arises, however, whether meningeal nerve endings were also stimulated. According to the description of Maximow & Bloom (1942) the dura and pia are richly supplied with nerves: apart from the extensive sympathetic nerve plexuses

there are sensory non-capsulated nerve terminations in the adventitia of the blood vessels; in addition, the cerebral dura and pia contain in their connective tissue numerous sensory nerve endings, some of which resemble Meissner's corpuscles; in the spinal pia similar nerve endings are also found but are very unevenly distributed. Clinical reports (for literature, see Pickering (1933)) indicate that stretching, but not cutting, the cerebral meninges gives rise to pain in the conscious subject; we are unaware of any similar observations on the spinal meninges in man. Our experimental findings considered in the light of the anatomical and clinical data (the latter, however, dealing almost exclusively with the brain) suggest that distension of the spinal subarachnoid space produces its effects reflexly on viscera and somatic muscles by mechanically stimulating afferents mainly in the posterior nerve roots; an additional factor may be traction on the spinal blood vessels. A direct action on the tracts in the spinal cord or on meningeal nerve endings would seem to be of minor or no significance.

Whenever we examined the effects of intrathecal injection of solutions, other than physiological saline, we used repeated saline control injections to determine the part played by mechanical factors in the total response. Before any injection, we generally withdrew an equal volume of cerebrospinal fluid or allowed the fluid to drip out till the normal rate of outflow was re-established.

Effects of intrathecal injection of KCl *solutions*

We have studied the effects of intrathecal injections of 0·1–0·2 c.c. of isotonic (1·15 %) and slightly hypertonic (1·3 %) KCl solutions and of solutions containing less than 1·15 % KCl which had been made isotonic by the addition of appropriate amounts of NaCl. Shanes (1946) points out that if the nerve fibre is permeable to K^+ but not to Na^+, a physiologically isotonic solution of increased K^+ concentration must consist of physiological saline with K^+ added to all its original electrolyte content. A solution in which Na^+ is simply replaced by K^+ is in effect hypotonic and causes swelling of the fibre. This factor was to some extent controlled by the frequent use of the slightly hypertonic KCl solutions. These KCl solutions regularly produced dilatation of the pupil, a rise of arterial blood pressure and various changes in the rate and rhythm of the heart, alterations in breathing and modifications of the spinal reflexes. Fig. 1 illustrates the results of a representative experiment. The mechanisms involved were analysed in detail.

Effects on the pupil

Intrathecal injection of a KCl solution in concentrations ranging from 0·13 to 1·3 % caused an immediate and conspicuous dilatation of the pupil; the dilatation usually began at the onset of the KCl injection, reached a maximum in about 5 sec. and declined more gradually in 20–40 sec. The pupil diameter

usually returned to normal when the blood pressure was still maintained at its peak level (Fig. 1). Sometimes the dilatation occurred in two phases, the first immediate and rapid, the second smaller and more gradual. The pupil dilatation occurred even while a strong white light was being shone into the eye to produce a background of maximal pupillary constriction.
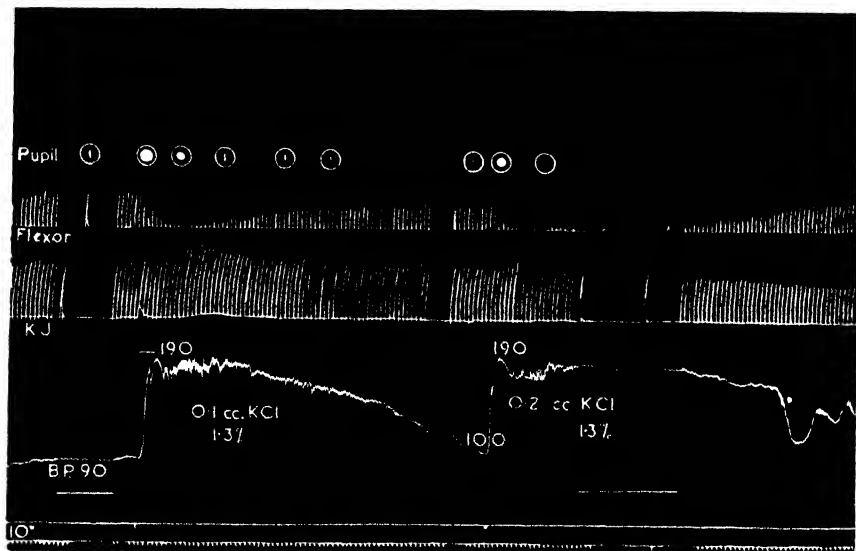
Fig. 1. Cat, 2·8 kg. Chloralose. Records from above downwards: pupil diameter (diagrammatic approximate representation); left flexor reflex recorded from tibialis anticus tendon; right knee jerk; carotid blood pressure; signal and time in 10 sec. 0·1 and 0·2 c.c. of 1·3% KCl were injected intrathecally at first and second signal respectively. The parts of the tracing that were recorded on the fast drum are underlined. Intrathecal injections were made at the level of the first sacral vertebra unless otherwise stated.

The following observations show that this pupillary dilatation is due to reflex inhibition of the III nerve nucleus. (1) The response is not produced by intravenous injection of equal or larger volumes of isotonic KCl solution. (2) It occurs after section of both cervical sympathetic nerves and removal of both adrenal glands. (3) It is not generally accompanied by contraction of the sympathetically innervated nictitating membrane. When the cervical sympathetic is stimulated with currents of decreasing intensity, the response of the dilator pupillae and of the nictitating membrane decline and finally disappear concurrently. If any threshold difference is observed the pupil response disappears before that of the nictitating membrane. (4) The response is abolished after intracranial section of the III nerve. The experiments were carried out as follows: the III nerve was cut on one (say right) side; the pupil dilated maximally. Eserine was then instilled into the right eye, producing maximal constriction of the pupil. The KCl solution was injected intrathecally;

the innervated left pupil dilated but the denervated right pupil was unaffected. Subsequent stimulation of the cervical sympathetic showed that the locally applied eserine had not raised the threshold for sympathetic dilatation of the pupil; in fact very weak stimuli, imperceptible when applied to the tongue, readily produced pupillary dilatation. (5) The latent period of the pupil response is so brief that there is no possibility of the intrathecally injected KCl solution having travelled up to the III nerve nucleus to exert an inhibitory action locally. (6) Direct application of the KCl solution to one of the lumbar posterior nerve roots (by methods to be described later) produces almost immediately reflex pupillary dilatation.

As already explained, the central inhibition of the III nerve nucleus set up by the impulses generated in the afferent nerves in the theca, can completely annul the central excitation contemporaneously produced in the nucleus by impulses resulting from a bright light falling on the retina.

It must be mentioned, however, that in one experiment intrathecal injection of a KCl solution produced in addition to pupil dilatation, well-marked contraction of the nictitating membrane, retraction of the lids and some apparent forward bulging of the eyeball; in this exceptional instance reflex stimulation of the sympathetic supply to the eye structures presumably occurred.

Effects on the circulation

Intrathecal injection of 0·1-0·2 c.c. of isotonic KCl solution caused a rapid increase in blood pressure; the pressure might rise by as much as 100 mm. Hg in 10 sec. The latent period was very brief, a few sec. (Figs. 1, 2 A). The blood pressure remained fairly steadily at its new high level for 4–5 min. and then began to decrease slowly, returning after 7–8 min. to approximately the control level. The liminal concentration of KCl in the injected solution needed to bring about the circulatory response was 0·065 %.

Cardiac changes. The changes in the rate and rhythm of the heart, were variable owing to the fact that two conflicting mechanisms are simultaneously in action, namely a sympathetic over activity due to the KCl injection and a cardiac slowing due to the rise of blood pressure. While the blood pressure is rising, and more regularly at its peak value, the heart is slowed for some time to a greater or less extent (Fig. 2 A). This slowing is probably the reflex result of the rise of blood pressure which stimulates the pressor receptors in the carotid sinus and aortic arch. The efferent pathway is mainly formed by the vagi, but even after double vagotomy the rise of blood pressure may sometimes produce a more transient and less marked initial cardiac slowing which is presumably due to reflex inhibition from the pressor receptors of cardiac sympathetic tone. In animals with intact vagi the cardiac slowing (just described) may be interrupted by bouts of rapid heart-rate and by occasional or multiple extra systoles; the latter are associated with the usual

transient drops in blood pressure. After double vagotomy (Fig. 2B) intrathecal injections of KCl more regularly produce at some stage a further increase in heart-rate, in addition to extra systoles. It would appear, therefore, that the cardiac quickening and extra systoles observed in the intact animal, and especially after vagotomy, are manifestations of over activity of the cardiac sympathetic nerves. The total effect on the heart-rate in any experiment thus depends on the relative importance of the reflex slowing, produced secondarily by the rise of blood pressure, and the increased cardiac sympathetic activity which is the immediate result of the KCl injection.

Analysis of the blood pressure changes. The rise of blood pressure occurs too rapidly and after too short a latency to be attributed with any probability to
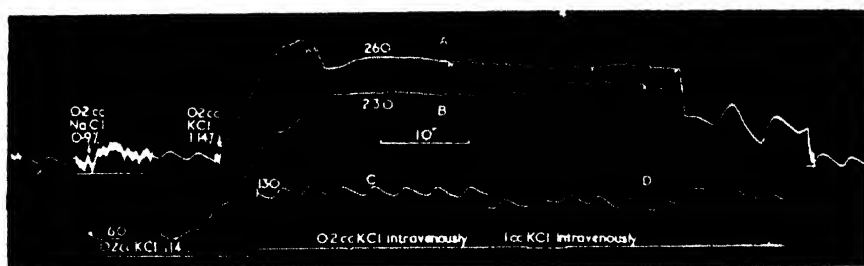
Fig. 2. Cat, 3·2 kg. Chloralose. Records of carotid blood pressure. A, preparation with vagi intact; at first arrow, inject intrathecally 0·2 c.c. 0·9% NaCl; at second arrow, inject intrathecally 0·2 c.c. 1·14% KCl. B, preparation with both vagi cut; at signal, inject intrathecally 0·2 c.c. 1·14% KCl. C, D, same preparation as in B; at signal, inject *intravenously* 0·2 c.c. and 1 c.c. 1·14% KCl. The parts of the tracing that were recorded on the slow drum are underlined. Time scale for parts of the tracing taken on the fast drum is given under tracing B.

a direct action on the circulation of KCl absorbed from the theca. Fig. 2 C, D shows that equal (0·2 c.c.) or much larger volumes (1 c.c.) of isotonic KCl solution rapidly injected intravenously have little or no pressor effect. The rise of blood pressure must be due to generalized vasoconstriction because of its magnitude, and because it may occur in spite of coincident marked cardiac slowing. It occurs after removal of both adrenal glands and so represents the result of increased discharge along vasomotor nerves. The integrity of the vasomotor centre is not essential as the pressor effect develops strikingly in animals in which the spinal cord has been transected in the upper cervical region. In some instances a contributory factor may be an increase in the venous return due to muscular spasm, but the rise of blood pressure can occur equally well in the absence of such spasm.

Mode of action of intrathecal KCl on circulation. The mode of production of the increased vasomotor and cardiac sympathetic discharge must next be considered. Intrathecally injected KCl may act (*a*) directly on the sympathetic neurones in the lateral horns of the spinal cord; (*b*) by stimulating the pre-

ganglionic sympathetic fibres in the anterior nerve roots; (c) reflexly, by stimulating nerve endings in the meninges, or nerve fibres in the posterior nerve roots or the white columns of the spinal cord.

The observations to be described show that the circulatory response is reflexly produced.

A direct action on the grey matter of the spinal cord or on the sympathetic outflow can be excluded in the following way. A solution of KCl was injected intrathecally at the level of the third lumbar vertebra in a caudal direction. The injected solution could thus not come into contact with or affect any segments of the spinal cord or the sympathetic outflow except possibly in L1 or 2. Diffusion of the KCl to these segments or roots from the site of the injection
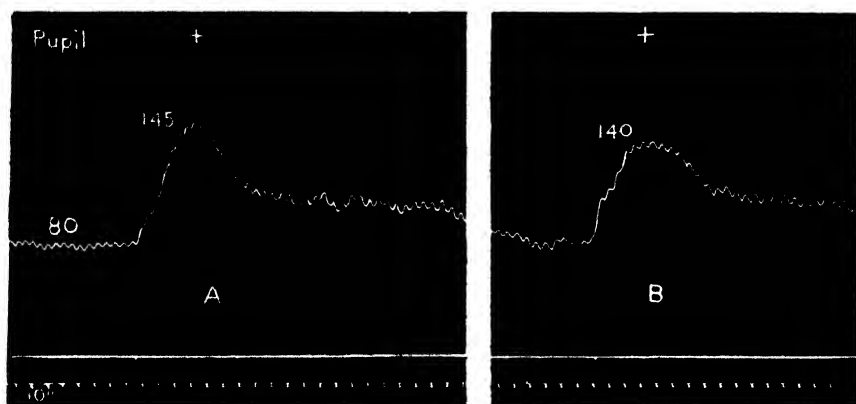
Fig. 3. Cat, 2·7 kg. Chloralose. Cat set up on a board in a vertical position. Injection needle introduced under the dura at L3. Dura opened at S1. A, needle pointing caudad; at signal, inject 0·5 c.c. 1·3% KCl. B, needle pointing cephalad; at signal, repeat injection of 0·5 c.c. 1·3% KCl. Pupil + represents dilatation of the pupil.

was further prevented by setting up the cats in a vertical position and by opening the dura at the level of the first sacral vertebra, so that the solution injected caudally at L3 would flow out at S1. This latter procedure likewise prevented any coincident rise of intrathecal pressure which might act directly or promote the flow of KCl to higher levels of the spinal cord. Fig. 3A shows that a caudally directed intrathecal injection of 1·3% KCl solution raised the arterial blood pressure. In this experiment a comparable rise of blood pressure occurred whether the injection was directed cranially or caudally; there was also well-marked pupil dilatation in both instances. In other instances, however, the cranially directed injection produced a greater pressor effect than the caudally directed injection.

Directly applied isotonic KCl solution does not stimulate the preganglionic fibres in the cervical sympathetic; there is thus no reason to suppose that it

would be effective on similar fibres in the anterior nerve roots. The rise of blood pressure resulting from intrathecal injection has a latency of only a few seconds which seems to allow insufficient time for the solution to penetrate through the spinal white columns into the lateral horns of the grey matter.

It seems therefore that the KCl solution must act on the afferent side of the reflex arc, i.e. on the posterior nerve roots themselves, on meningeal nerve endings or on afferent tracts in the spinal cord. It has been conclusively shown below that isotonic or weaker KCl solutions stimulate directly afferent fibres in the posterior nerve roots.
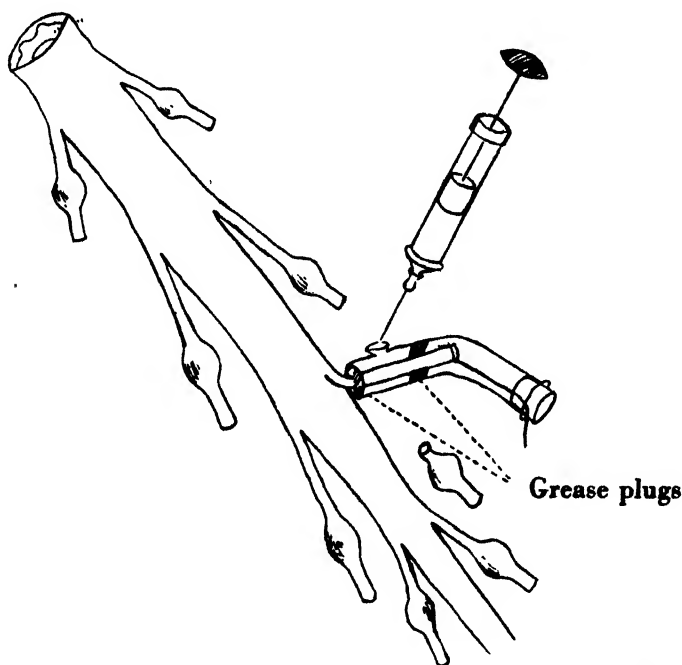
Fig. 4. Diagram illustrating the 'posterior nerve root preparation'. For explanation see text.

Effects of KCl *application on posterior nerve root preparation*

The seventh lumbar root was generally selected for examination because of its length. It was tied, divided just centrally to the junction with its ganglion, and dissected carefully as far as its attachments to the spinal cord. The cut root was then introduced into a narrow glass tube and the KCl solution was injected through a small opening on the upper surface. The arrangement finally employed is shown in Fig. 4. The two grease plugs prevent the escape of the solution which can thus be left in contact with the root for predetermined periods: the solution is also prevented from making contact with the injured region of the nerve at the site of the ligature. The action of KCl on normal posterior nerve root fibres can thus be studied.

Control experiments were always carried out first with isotonic saline and then followed by the application of KCl solutions of various strengths. The volume employed varied from 0·05 to 1·5 c.c. In general the NaCl solution produced negligible reflex effects; KCl solutions on the other hand regularly produced a reflex rise of blood pressure and dilatation of the pupil. Representative results are illustrated in Fig. 5. A control application of isotonic saline was without effect. After 1 min. the saline was withdrawn by aspiration and replaced by an equal volume (1 c.c.) of isotonic KCl. There was an almost immediate dilatation of the pupil followed after a few seconds by a quick rise of blood pressure from 130 to 185 mm. Hg with a further slower rise to 195 mm. Hg. After 1 min. the KCl solution was aspirated and the blood pressure
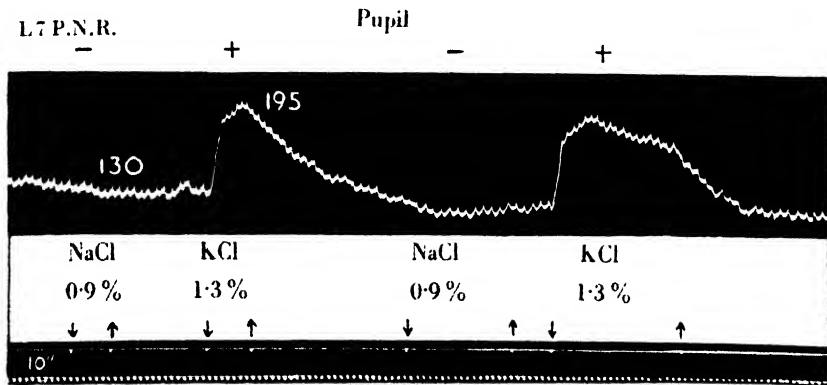
Fig. 5. Cat, 2·9 kg. Chloralose. Seventh lumbar posterior nerve root preparation. Above: record of carotid blood pressure and pupil diameter (+ indicates dilatation, − no change in pupil diameter). Below: signal and time in 10 sec. Between first two arrows, apply 0·9% NaCl to the posterior nerve root; at second arrow, the NaCl solution was removed by aspiration; between third and fourth arrows, apply 1·3% KCl (this solution was removed at fourth arrow); between fifth and sixth arrows, repeat application of 0·9% NaCl; between seventh and eighth arrows, repeat application of 1·3% KCl.

gradually declined, returning to control level in 4 min. After another control saline application, the KCl solution was applied to the posterior nerve root for about 3 min. The blood pressure tended to be sustained near its peak value, but on aspirating the KCl solution, the pressure began to fall fairly rapidly. It is certain therefore that KCl stimulates certain afferent fibres in the posterior root. In the absence of electrical recordings we cannot say for how long the afferent volleys are maintained, as the duration of the efferent discharges (as judged by the blood pressure and pupil diameter) is modified by central processes like after-discharge. The more sustained effect obtained with longer applications of KCl suggests that one is dealing with maintained trains of volleys rather than with a brief group of volleys. The weakest solution effective on the root was an isotonic solution containing 0·13% KCl; no response was obtained when a 0·065% KCl solution was applied.

The effects of intrathecal injection of KCl on pupil and blood pressure are sometimes dissociated, i.e. pupil dilatation occurring without a rise of blood pressure, and vice versa. As the central pathways and relay stations of the two reactions are quite distinct, it is not surprising to find that at times transmission may take place more readily through one reflex arc than the other. Similarly, the fact that the pupillary response usually subsides long before the pressor effect may likewise be due to differences in such central properties as after-discharge.

Effect of KCl *application on peripheral nerve trunks*

If an isotonic KCl solution is applied to a peripheral nerve (e.g. sciatic, saphenous) no visceral reflex effects or peripheral actions on skeletal muscle are observed. If the connective tissue is dissected off and the component nerve bundles separated out, subsequent application of KCl solution (directly, or with the nerve in a tube) may produce considerable pupillary dilatation but only a modest blood pressure rise (e.g. 20 mm. Hg). The pressor response is always smaller than that elicited from a single posterior nerve root. The possible reasons for this difference are considered in the discussion.

Action of KCl *on nerve endings*

There is a good deal of evidence that KCl can stimulate nerve endings. Moore, Moore & Singleton (1934) and Moore (1934) found that KCl in concentrations down to 0.35% injected intra-arterially in cats produced pain, as judged by pseudaffective reactions; the nerve endings involved were localized in the vicinity of the capillaries. Intradermal injections in man of potassium phosphate are more painful than sodium phosphate (Habler & Hummel (1928)). Brandi (1931) thought that spinal anaesthetics are more liable to cause headache if the K^+ content exceeded 10 mg. %. In conformity with these results we have shown in experiments in man that injection of small volumes (0·05 c.c.) of isotonic KCl solution into the skin or into skeletal muscles produces an immediate sharp severe pain which may last for several (5–15) minutes. The effects contrast strikingly with the trivial discomfort produced by injections of corresponding volumes of isotonic saline. Isotonic $CaCl_2$ solution is also painful on subcutaneous or intramuscular injection in man, but less so than KCl.

In chloralosed cats, subcutaneous or intramuscular injections of isotonic KCl solution or its application to the cornea elicited no reflex responses. We have from time to time obtained blood pressure and pupil responses by painting isotonic KCl solutions on the outer surface of the exposed spinal meninges. The effects have not been striking and we could not exclude the possibility that they were due to the KCl penetrating through the meninges to act on the posterior nerve roots themselves. We have examined the effect of intra-

thecal KCl injections after painting the external surface of the dura with 2 % procaine. The results are not easy to interpret as the procaine can penetrate ultimately to the roots and to the spinal cord itself. We have noticed, however, when the dura is no longer responsive to weak electrical stimulation, that intrathecal KCl is still active, though to a diminished extent, on blood pressure and is less active or inactive as far as the pupil is concerned. One cannot argue from the fact that because a drug acts on pain receptors in one locality that it will act on receptors of undetermined character elsewhere. Thus, as mentioned above, a $CaCl_2$ solution stimulates pain receptors in man, and as shown by Moore (1934), acts similarly on perivascular pain receptors in cats. We have shown (see p. 224) that directly applied $CaCl_2$ solution does not stimulate the posterior nerve root preparation; as intrathecally injected $CaCl_2$ does not elicit reflex effects it would appear not to act on meningeal receptors either.

A comparison of the results of intrathecal injection of KCl and of application of a KCl solution to one or more posterior nerve roots suggests that quantitatively as well as qualitatively the effects of intrathecal KCl injection on the circulation and pupil can be fully accounted for by the demonstrated results of stimulation of the posterior roots by KCl. In the absence of more direct evidence we are inclined to attribute little if any role to an action of KCl on the meningeal nerve endings as far as the visceral reactions described are concerned.

We conclude from the experiments described and the reasons adduced that the circulatory (like the pupillary) response to intrathecal injection of KCl solutions is produced reflexly mainly (if not wholly) as a result of stimulation of posterior nerve root fibres; the efferent pathway is the sympathetic outflow from the thoracic and lumbar segments of the spinal cord to the heart and blood vessels. The rise of blood pressure may secondarily produce reflex cardiac slowing via the pressor receptors.

Effects on respiration

When a solution of KCl is injected into the sacral or lumbar region of the theca respiration is usually not much altered. There is commonly and initially a long drawn inspiration followed by a pause, after which breathing returns to its control state. As similar effects are produced when a KCl solution is applied to a posterior nerve root, they are reflexly produced. We have found, however, that when the intrathecal injection is made at a higher level, e.g. at cervical 4–6, respiratory movements are depressed or may cease. Thus, in one experiment, injection of 0·2 c.c. of isotonic KCl caudally at the level of C5–6 caused an immediate slowing of respiration and a slight decrease in depth; as the associated blood-pressure rise passed away breathing became slower and deeper. After a second injection the breathing became very slow and deep

and ultimately stopped. In another experiment, injection of 0·5 c.c. of isotonic KCl at this level caudally produced an initial strong prolonged inspiratory spasm ('apneusis') followed by a short series of gasps and soon by respiratory arrest. In neither experiment was natural breathing restored by a prolonged bout of artificial respiration. These results have not yet been further examined, but they resemble those observed after intracisternal injections of KCl (Walker, Smolik & Gilson, 1945).
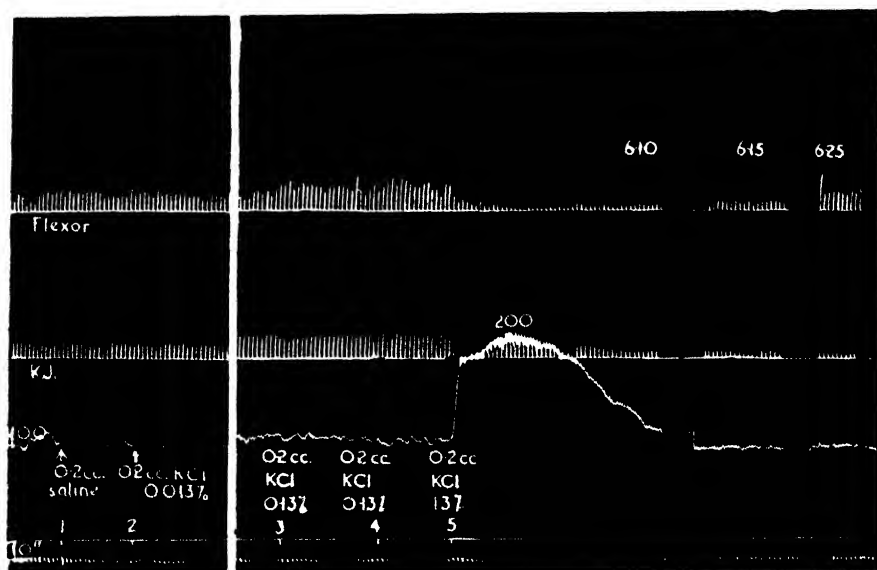
Fig. 6. Cat, 2 kg. Chloralose. Records from above downwards: left flexor reflex; right knee jerk; carotid blood pressure; signal and time in 10 sec. Inject intrathecally at 1, 0·2 c.c. 0·9% NaCl; at 2, 0·2 c.c. 0·013% KCl; at 3, 0·2 c.c. 0·13% KCl; at 4, 0·2 c.c. 0·13% KCl; and at 5, 0·2 c.c. 1·3% KCl. At 6·10 and 6·15 the drum was stopped for 5 and 10 min. respectively.

Effects on spinal reflexes

We have examined the effects of intrathecal KCl injections on muscle tone, knee jerk, and flexor reflex.

The *flexor reflex* was regularly depressed (Figs. 1, 6); in some cases the reflex was completely abolished (even when maximal afferent stimulation was employed) for periods as long as 20 min. The depression may set in gradually, reaching its maximum after some minutes or it may develop abruptly, the reflex disappearing in 10–20 sec. Occasionally there is a short-lived initial rise of resting flexor tone.

The *knee jerk* may be potentiated or depressed (Figs. 1, 6); quadriceps muscle tone may be increased. Fig. 1 shows that after intrathecal KCl injection there was a transient, but considerable, increase in quadriceps tone;

this was accompanied by a brief extension of the legs and contractions of the muscles of the back. There was some increase in the height of the knee jerk as measured from the base line of raised 'resting' quadriceps tone. Quadriceps tone then gradually returned to control level, while the knee jerk was further enhanced; it also became less pendular in character, i.e. the after-swing below the base line disappeared, indicating that the quadriceps muscle was relaxing more slowly presumably owing to prolonged central after-discharge. After $3\frac{1}{2}$ min. the knee jerk was back to its control state.

We have studied the effects on the flexor reflex of application of KCl solutions to the posterior nerve root preparation; we were unable for technical reasons to examine the effects of this procedure on the knee jerk. When the seventh lumbar root on the same or opposite side was so treated the flexor reflex was unaffected; sometimes, however, extensor movements were noted in the limbs. Application of KCl to the posterior root of L6, S1, 2 or 3, or to all these roots together on the same or opposite side, likewise did not affect the flexor reflex.

The observations of Mullins, Hastings & Lees (1938) throw some light on the interpretation of our results. They studied the effects on the muscles of the hind limbs (electrical recording) of altering the K/Ca ratio in the fluid bathing the lower spinal cord in dogs. When the posterior roots of the lumbar spinal cord were 'washed' (details are not given of the exact procedure used) with a solution free from Ca^{++} and high in K^+ there was no induced activity in the limb muscles; there was likewise no response when the lower spinal cord was irrigated with this solution. Mullins *et al.* did not examine the effects of these procedures on the circulatory responses. These results support our contention that high K^+ concentrations act less readily on the afferents affecting somatic muscle than on those reflexly affecting viscera.

Another point to bear in mind is that if the changes caused by intrathecal KCl solutions on the somatic reflexes were produced wholly reflexly, one would expect the law of reciprocal innervation to apply; one should thus find that depression of the flexor reflex was accompanied by enhancement of the extensor reflexes. Such relationship has, by no means, been generally observed; e.g. in Fig. 6 both the flexor reflex and the knee jerk were simultaneously depressed.

When the seventh lumbar posterior nerve root was stimulated electrically, to produce a rise of blood pressure and pupil dilatation approximately equal to that resulting from the application of a KCl solution, there were violent generalized movements of the chloralosed animal, although in the same preparation application of KCl to the root produced only trivial somatic effects. It would seem that while KCl picks out in the main the afferents in the posterior root concerned with *visceral* responses, electrical stimulation also simultaneously affects the afferents responsible for *somatic* reactions; our

argument implies that different afferents are responsible for the production of visceral responses and for changes in the somatic reflexes. We are thus unable to account fully for the changes in the somatic reflexes resulting from intra-thecal injection of KCl in terms of stimulation of posterior nerve roots alone; in fact the evidence suggests that their role may be comparatively unimportant. Other sites of action of KCl must therefore be considered, including the spinal cord itself and the nerve-endings round the spinal blood vessels.

Effects of intrathecal injections of $CaCl_2$

Intrathecal injection of $CaCl_2$ in isotonic concentration has no effect on the blood pressure, pupil or spinal reflexes. $CaCl_2$, however, antagonizes the action of KCl by diminishing or abolishing on the *afferent* side the effects of intrathecal injections of KCl. This conclusion is derived from experiments carried out as follows: KCl was first injected intrathecally; the usual effects appeared (rise of blood pressure, dilatation of pupil, effects on spinal reflexes). When they were fully established, 0·1–0·2 c.c. of isotonic $CaCl_2$ solution was injected intrathecally: commonly the rise of blood pressure due to KCl was cut short and the inhibition of the flexor reflex was annulled, the reflex responses returning gradually to their pre-injection level. When $CaCl_2$ was injected intrathecally·together with KCl, either mixed up in the same syringe or at an interval of about 5 sec., the familiar effects brought about by KCl did not appear or were greatly diminished in comparison with those obtained with KCl alone.

The antagonistic actions of $CaCl_2$ and KCl are most convincingly demon-strated on the posterior nerve root preparation. Isotonic $CaCl_2$ solution applied directly to the root produces no reflex effects. Likewise, if an isotonic $CaCl_2$ solution is applied mixed with an equal volume of an isotonic KCl solution, it may be without effect (Fig. 7) and, further, it may for some minutes afterwards annul the effects of a simple application of KCl alone. Thus, in Fig. 7, application of 1 c.c. of 1·3 % KCl solution to the posterior nerve root reflexly raised the blood pressure by 50 mm. Hg; application of 0·5 c.c. of isotonic $CaCl_2 + 0·5$ c.c. of 1·3 % KCl had no effect. The mixture was withdrawn by aspiration; 1 c.c. of 1·3 % KCl solution was then applied and aspirated twice without effect; but the third, fourth, and fifth application of KCl reflexly produced in-creasingly marked pressor responses.

Application of 3 % sodium citrate or of a mixture of 3 % sodium citrate and 2 % 'acid citrate' at pH 7·4 to the seventh lumbar root had no reflex effects (in spite of the decrease in concentration of ionized Ca^{++}) and did not facilitate the responses to subsequently applied KCl solution.
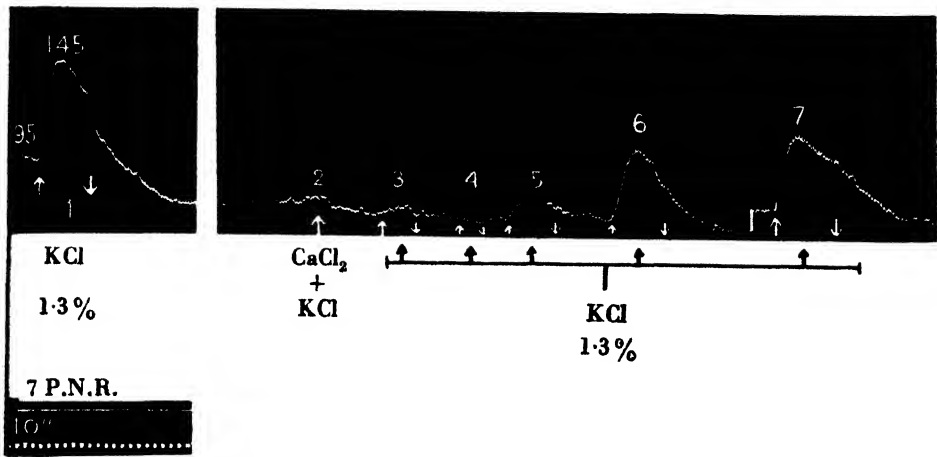
Fig. 7. Cat, 3 kg. Chloralose. Seventh lumbar posterior nerve root preparation. Carotid blood pressure and time in 10 sec. During 1, apply 1·3% KCl to L7 posterior nerve root; during 2, apply 1·2% CaCl₂ mixed with an equal volume of 1·3% KCl; during 3, 4, 5, 6, 7 apply 1·3% KCl.
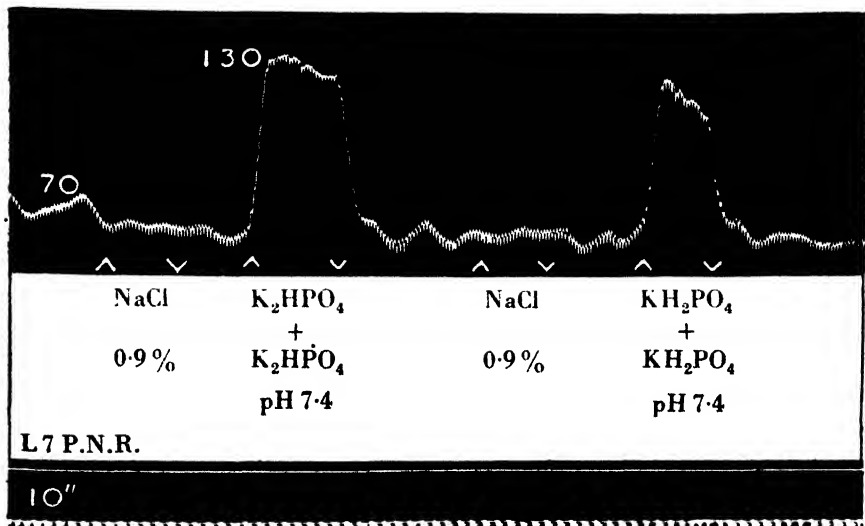
Fig. 8. Cat, 3 kg. Chloralose. Seventh lumbar posterior nerve root preparation. Records of carotid blood pressure and time in 10 sec. At first arrow, apply 0·9% NaCl which is removed at second arrow; at third arrow, apply 'Stern mixture' which is removed at fourth arrow; at fifth arrow, apply 0·9% NaCl which is removed at sixth arrow; at seventh arrow, apply 'Stern mixture' which is removed at eighth arrow.

Effect of intrathecal injections of other potassium salts

Stern and Chvoles (1933) and Stern (1942 *a*, *b*) have shown that intracisternal and intraventricular injections of an isotonic mixture of KH_2PO_4 and K_2HPO_4 at pH 7·4 cause a rise of blood pressure of considerable duration. They attribute this response to a direct action of potassium salts on the vasomotor centre. We found that 0·1–0·2 c.c. of such mixtures injected intrathecally at the level of the sacral or lower lumbar segments, produce a striking sustained rise of blood pressure, accompanied by a long-lasting dilatation of the pupil. We are under the impression that these responses are of greater duration than those following equivalent injections of KCl solutions. The pressor effect is not annulled by high transection of the spinal cord. When the same solution is applied to the posterior nerve roots (Fig. 8) there is a striking reflex rise of blood pressure and the pupil dilates. The effects of intrathecal injection of the 'Stern solution' on the blood pressure and pupil can thus be fully accounted for by direct stimulation of the posterior nerve roots.

Effect of intrathecal injections of hypertonic NaCl solutions

An intrathecal injection of 0·1–0·5 c.c. of a 2–5 % solution of NaCl causes effects on the blood pressure, pupil and spinal reflexes similar to those already described for KCl (Figs. 9, 10). Intrathecal hypertonic solutions of NaCl act on the blood pressure very rapidly and with short latency. The speed and extent of the blood-pressure rise is about the same as with KCl solutions, i.e. 70–100 mm. Hg. The pupil dilatation too occurs early, sometimes during the injection or immediately after.

Using the posterior nerve root preparation we have found that the action of intrathecal hypertonic saline on the blood pressure and pupil can be fully accounted for by impulses set up by the solution in the posterior nerve roots (Fig. 11) which reflexly stimulate the sympathetic cells in the lateral horns (raising the blood pressure) and reflexly inhibit the III nerve nucleus (dilating the pupil).

The spinal reflexes are modified by intrathecal hypertonic NaCl in a similar way as by KCl. The flexor reflex is inhibited or abolished (Fig. 9) while the knee jerk is variably affected (Fig. 10).

Effects of intrathecal injections of other substances

Hypertonic solutions of glucose and urea caused smaller effects on the blood pressure and pupil than saline solutions of equivalent hypertonicity (Fig. 10). It is interesting to recall that Verney (1946) found that hypertonic solutions of urea and glucose given by intracarotid injection are less efficient than saline solutions of equivalent strength in stimulating the osmoreceptors which control the secretion of the posterior pituitary.

Acid saline (pH 6–3) and alkaline saline (pH 8–10) were intrathecally injected but caused no changes in the blood pressure, pupil or spinal reflexes. Alkaline saline, at pH 9·5–10, caused an immediate great rise of blood pressure, dilatation of the pupil, and an increase of the tone of the extensor muscles of the legs (Fig. 12).
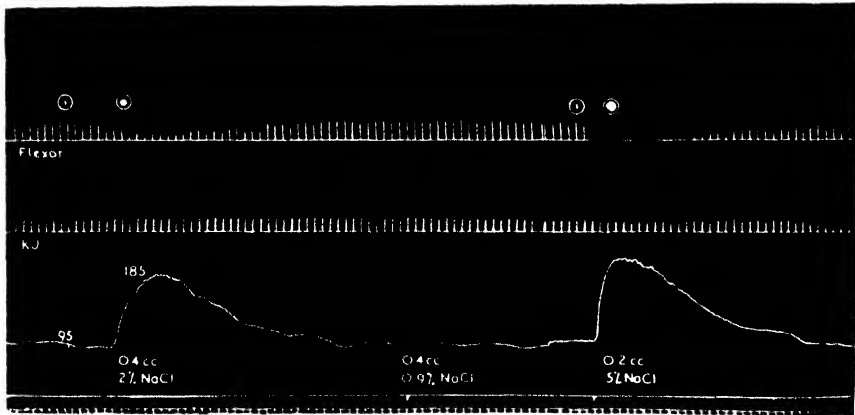
Fig. 9   Cat. 2·5 kg. Chloralose. Records from above downwards: pupil diameter (diagrammatic representation); left flexor reflex; right knee jerk; carotid blood pressure; signal and time in 10 sec. At first signal, inject intrathecally 0·4 c.c. 2% NaCl; at second signal, inject intrathecally 0·4 c.c. 0·9% NaCl; at third signal, inject intrathecally 0·2 c.c 5% NaCl.
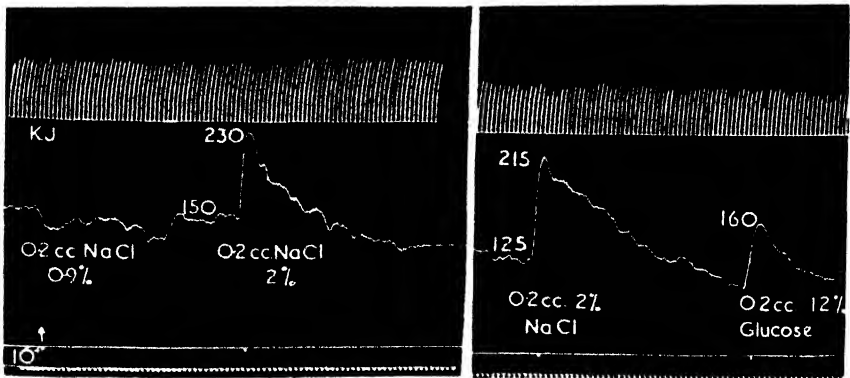
Fig. 10. Cat, 2·6 kg. Chloralose. Records from above downwards: knee jerk, blood pressure; signal and time in 10 sec. At arrow, inject intrathecally 0·1 c.c. 0·9% NaCl; at second signal, inject intrathecally 0·2 c.c. 2% NaCl; at third signal, inject intrathecally 0·2 c.c. 2% NaCl; at fourth signal, inject intrathecally 0·2 c.c. 12% glucose.

Eserine and prostigmine even in very high concentrations (1 : 1000) did not produce any effect on the blood pressure and pupil when applied to the central end of the cut posterior root, or when intrathecally injected (when mechanical
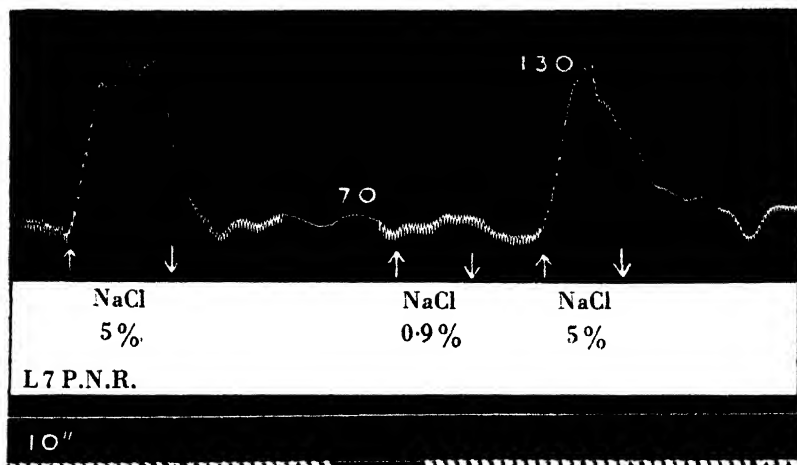
Fig. 11. Cat, 3 kg. Chloralose. Seventh lumbar posterior nerve root preparation. Carotid blood pressure and time in 10 sec. Between first and second arrow, apply 5% NaCl to L7 posterior nerve root; between third and fourth arrows, apply 0·9% NaCl; between fifth and sixth arrows, apply 5% NaCl.
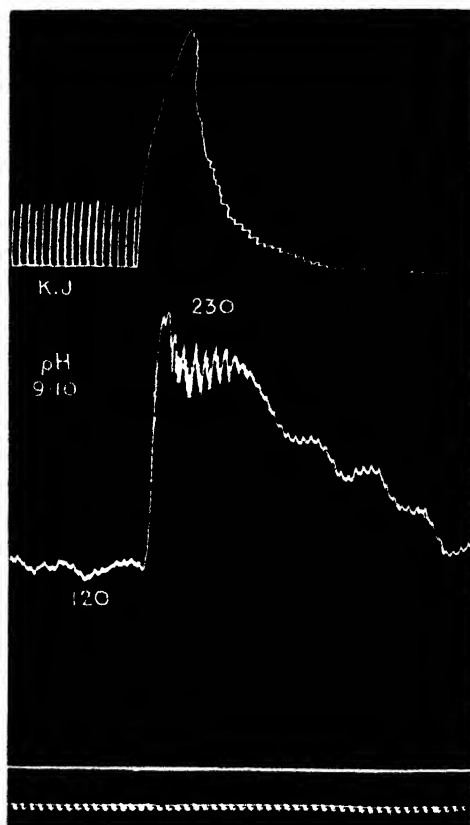
Fig. 12. Cat, 2·5 kg. Chloralose. Records from above: knee jerk; blood pressure; signal and time in 10 sec. At signal, inject intrathecally 0·2 c.c. 0·9% NaCl at pH 9–10.

effects were avoided). Acetylcholine (in concentrations as high as 1 : 1000) was similarly ineffective, when applied to posterior nerve roots.

Intrathecal injection of 0·5 c.c. of heparinized blood taken freshly from the same animal, or of heparinized blood (taken from another animal) that had been allowed to stand overnight, or of human stored blood was without effect.

DISCUSSION

Several matters arising out of the results just recorded seem to merit further consideration. They are: (1) the action of K^+ on nerve fibres in general; (2) the particular fibres in the posterior nerve root acted on by K^+ in our experiments; (3) a comparison of the results of injecting K^+ into the lower spinal theca and into the cisterna magna or cavity of the ventricles.

Action of potassium on nerve fibres

It has long been known that potassium-rich solutions exert a complex influence on axons. Fenn (1940), summarizing the literature, states that the threshold of electrical stimulation is in general first lowered and then raised by K^+; larger amounts of K^+ finally cause inexcitability. There has, however, been a tendency to stress the depressant rather than the excitatory effects of K^+, and in some recent studies the former alone were observed. Thus Rosenberg & Kitayama (1929) found that when frog sciatic nerve is bathed in an isotonic Ringer's solution containing four times the normal KCl concentration, the height, rate of rise and velocity of the action potential are noticeably reduced after 30 min. These changes are more clearly and rapidly produced by solutions containing ten times the normal K^+ concentration. Cowan (1934) showed in Maia nerve that KCl rich solutions rapidly and reversibly depressed both the action current and the injury potential; he attributed the reversible inexcitability which occurs in freshly dissected crab nerve to the presence of an abnormally high concentration of K^+ salts on the outside surfaces of the fibres. Similarly Hertz (1945) found an immediate reduction in the amplitude of the action potential on immersing single frog nerve fibres in an isotonic solution containing six to ten times the normal concentration of K^+.

The stimulating action of K^+ on axons is, however, at least equally well established. Thus Cicardo (1939) produced contraction of the gastrocnemius muscle by injection of a KCl solution into the sciatic nerve. Brown & MacIntosh (1939), using the method of intra-arterial injection, found that an isotonic KCl solution set up a repeated discharge of impulses from nerve fibres in their continuity; positive results were obtained from various motor and sensory somatic nerves and from preganglionic autonomic nerves. An isotonic solution containing KCl and $CaCl_2$ in the proportion of 1 : 2 did not evoke discharges. Hodgkin & Huxley (1945) found that a drop of isotonic KCl solution placed on the axon of the squid caused a transient discharge of impulses and a steady resting potential between the stimulated and a resting point; KCl produced local

voltage changes similar to those resulting from cutting the fibre or crushing it with forceps or from the local application of chloroform. Lehmann (1937 *a*, *b*), using cat nerve, found that excess K^+ (or alkalinity or lack of Ca^{++}) decreased the negative after-potential, increased the positive after-potential and caused 'spontaneous' discharges. Brink, Sjostrand & Bronk (1938 *a*, *b*, 1939), using single fibres of frog sciatic nerve previously soaked in NaCl solution (to remove Ca^{++}), obtained activity, associated with a negative demarcation potential, with a solution containing four times the normal KCl concentration. When the fibre had been stimulated to discharge by sodium citrate solutions, the addition of low concentrations of KCl produced an initial increase in discharge frequency, followed by a secondary decline. Larrabee, Brink & Bronk (1939) found that high K^+ concentrations stimulate preganglionic axons. It is difficult to generalize from the evidence just quoted about the exact conditions which determine whether the excitatory or depressant action of K^+ shall predominate in any particular type of experiment. Our own observations on the 'posterior nerve root preparation' demonstrate quite clearly that isotonic or weaker solutions of KCl or potassium phosphate stimulate certain afferent fibres in these roots, an effect which, in conformity with the results of Brown & MacIntosh (1939) and others, is annulled by Ca^{++}. In our hands, using the method of external application, the excitation is more strictly limited as regards the type of fibre affected than was the case with other workers; thus we failed to stimulate preganglionic fibres and produced only slight effects on peripheral nerve trunks.

Action of potassium on posterior nerve roots

We must next consider which components of the posterior nerve roots are stimulated in our experiments. These roots contain numerous non-medullated afferent fibres which far exceed the medullated fibres in number and which have their nutrient cells in the root ganglion (Ranson, 1911, 1912 *a*, *b*). They probably mediate pain sensibility (Ranson, 1943). A cut which divides the lateral division of the posterior root (e.g. of L7) as it enters the spinal cord, without injury to the medial division, eliminates the pain reflexes, consisting of struggling, acceleration of respiration and rise of blood pressure (Ranson & Billingsley, 1916; Ranson, 1943) which are normally elicitable from the root in the anaesthetized cat; the lateral division is found to contain the non-medullated fibres. Section of the medial root on the other hand, has little effect on the pain reflexes. There is an obvious resemblance between the reflex responses that depend on the integrity of the non-medullated fibres in the lateral division and those which we produced by direct application of KCl to the posterior root; we are inclined to conclude that it is in fact the non-medullated fibres in the posterior root which are stimulated by KCl. As we emphasized in the text it is quite certain that not all the posterior root

afferents are stimulated by KCl as the response obtained tends to be restricted to the viscera and (unlike electrical stimulation of the root) affects the somatic muscles only to a limited degree, if at all. The absence of a sheath in the non-medullated fibres may be one reason why they are acted on selectively, but it cannot be the whole explanation. The non-medullated fibres under discussion are all derived from the peripheral nerves and are found in large numbers in purely afferent nerves (e.g. saphenous) or mixed nerves (e.g. sciatic). But application of KCl to these peripheral nerves has proved comparatively ineffective in our hands in spite of their high content of non-medullated fibres. Preliminary dissection of the peripheral nerves (removal of the connective tissue sheath, separation of the constituent nerve bundles) somewhat facilitates the excitatory action of KCl on them. The fact that the non-medullated fibres in the root are superficially placed and sheathed in little connective tissue may increase their susceptibility to the action of KCl.

The discrepancy between the reflex response to KCl on the root and on the 'fasciculated' peripheral nerve may be due, however, to an additional factor. Sherrington (1898), in his classical description of spinal shock, states: 'Excitation, mechanical or by weak currents quite imperceptible to the tongue of the *central ends of the spinal posterior (dorsal, sensory) roots themselves, fairly readily evokes the usual reflex movements* elicitable—although enormously stronger stimuli°fail absolutely when applied to the skin and afferent nerve trunks.' (Italics in the original.) Judged by effector (in this case somatic muscle) reactions, stimulation of a few posterior root fibres is thus more effective than stimulation of all the afferent fibres in large peripheral nerves. The discrepancy is not yet fully accounted for and has been attributed (i) to the root containing many more pain fibres than a peripheral nerve (Denny-Brown, 1939), or (ii) to the fact that as the root stimulus is applied close to the cord a synchronized volley is set up which on reaching the grey matter produces more effectively a threshold of central excitatory state. Both explanations are open to objections, but the fact remains that posterior root stimulation more readily evokes reflex responses than does afferent stimulation of peripheral nerves. All the various reasons mentioned above seem together to account adequately for the far greater effectiveness of action of KCl on posterior roots than on peripheral nerves. Brown & MacIntosh (1939), however, by the method of intra-arterial injection, successfully obtained discharges from a much wider range of nerve fibres than we could by direct application of KCl.

Comparison of effects of intrathecal (spinal) and intracisternal or intraventricular injections of KCl and other solutions

The studies of Stern & Chvoles (1933), Resnik, Mason, Terry, Pilcher & Harrison (1936), Mullins *et al.* (1938), Stern (1942 *a*, *b*), Downman & Mackenzie (1943), Euler (1938) and Walker *et al.* (1945) provide a full survey of the effects

of intracisternal and intraventricular injections of K$^+$ salts and of modifications in the electrolyte balance produced in other ways in the cerebrospinal fluid in the region of the medulla oblongata. When a solution with an abnormally high K/Ca ration (due to excess K$^+$ or deficient Ca^{++}) is injected intracisternally in dogs (Mullins *et al.* 1938) there is initially increased muscular activity (tetany, dorsal curvature of the tail, extensor rigidity of the legs, contracted abdominal muscles) and stimulation of respiration, followed by a rise of blood pressure and cardiac slowing; after curari, which abolishes the somatic muscular activity, the circulatory changes are less marked. Both Downman & Mackenzie (1943) and Walker *et al.* (1945) confirm the observation of Resnik *et al.* (1936) that small doses of KCl usually produce an initial *fall* of arterial blood pressure (which may be substantial, e.g. from 120 to 50 mm. Hg) associated with slowing of the heart; larger doses tend to give a small initial depression and are followed commonly, but not regularly, by a well-marked rise of pressure. The rise reaches its peak slowly (e.g. in 3·5 min.) after a latency of 0·5–1 min. In addition to respiratory stimulation there may be signs of respiratory failure (e.g. apneuses) associated with circulatory collapse. The results summarized above of raising the K$^+$ concentration or the K/Ca ratio in the cerebrospinal fluid in the region of the medulla oblongata thus differ in obvious and important respects from those obtained by us by injecting K$^+$ into the lower spinal theca. Compared with low intrathecal injection, intracisternal injection produces its circulatory effects after a longer latency (minutes instead of seconds), it frequently produces an initial fall of blood pressure (instead of the invariable rise) and the circulatory changes are longer lasting; respiration also is more frequently disordered and respiratory and circulatory failure is common. The effects of intracisternal injection are generally and reasonably attributed to an action, first stimulant and then depressant on the various medullary centres, vasoconstrictor, vasodilator, cardio-inhibitory, cardio-accelerator, respiratory (inspiratory, expiratory, 'pneumotaxic' and suprasegmental somatic). We have, however, already demonstrated that the effects of spinal intrathecal injections of K$^+$ on the circulation, respiration and pupil are *reflexly* evoked and that the circulatory responses are independent of the integrity of the medullary centres. Our results suggest that with intracisternal injection, in addition to the direct action on the medullary centres, stimulation of afferent nerves may play some contributory part. On the other hand, the depression of respiration which we noted with *cervical* injections of K$^+$ may be due to a central action.

SUMMARY

1. Fluids of various composition and volume were injected intrathecally in cats under chloralose anaesthesia, usually at the level of the first sacral vertebra.

2. Injection of 0·9 % NaCl at pH 7·4 in volumes of 0·5 c.c. or over, especially under conditions where a rise of fluid pressure takes place in the subarachnoid space, produces reflexly a rise of arterial blood pressure and dilatation of the pupil. The results are probably mainly due to mechanical stimulation of the posterior nerve roots.

3. Intrathecal injection of 0·1–0·2 c.c. of isotonic (1·15 %), slightly hypertonic (1·3 %) solutions of KCl, or of solutions containing less than 1·15 % KCl, but made isotonic by adding NaCl, regularly produces dilatation of the pupil, a rise of arterial blood pressure, changes in the rate and rhythm of the heart, alterations in breathing, and modifications of the spinal reflexes.

4. The KCl solutions employed specifically stimulate by virtue of their potassium ions afferent fibres (probably non-medullated) in the posterior nerve roots. The afferent impulses so set up: (i) reflexly dilate the pupil by inhibition of the III nerve nucleus (the sympathetic pupil dilators are rarely involved); (ii) reflexly produce vasoconstriction via the sympathetic outflow to the blood vessels; the rise of pressure occurs after removal of the adrenals and after transection of the spinal cord in the mid-thoracic region (the vasomotor centre is thus not indispensable for the response); (iii) reflexly produces acceleration and increased excitability of the heart mainly by stimulation of the cardiac sympathetic fibres; the rise of blood pressure, via the pressoreceptors, reflexly tends to slow the heart.

5. Application of KCl solutions to the 'isolated posterior nerve root preparation' produces similar changes to those described in (4). KCl is far less effective when applied to peripheral nerves; the reasons for this difference are discussed.

6. Isotonic KCl solutions injected into skin or muscle in man stimulate pain nerve endings.

7. The effects of intrathecal injections of KCl on breathing are in part reflexly produced. Injection of KCl at higher spinal levels (e.g. lower cervical) may produce respiratory (and circulatory) failure probably owing to a central depressant action.

8. Intrathecally injected KCl regularly depresses the flexor reflex and has variable effects on the knee jerk. These changes are not reproduced by application of KCl to the 'posterior nerve root preparation'. It is suggested that externally applied K^+ specifically stimulates the afferent fibres in the posterior roots concerned with visceral reflexes, but affects less, if at all, the afferents concerned with somatic reflexes.

9. Mixtures of KH_2PO_4 and K_2HPO_4 in isotonic strength at pH 7·4 produce similar effects (both intrathecally and on the posterior root preparation) to those of KCl (as described in (4)).

10. $CaCl_2$ annuls the stimulating action of KCl on the posterior nerve root.

11. Hypertonic solutions of NaCl, urea or glucose stimulate the posterior nerve roots and produce the same visceral effects as isotonic KCl.

12. The effects of intrathecal injection of acid and alkaline solutions, eserine, acetylcholine and blood are briefly noted.

13. The literature dealing with the action of K^+ on nerve fibres is reviewed; there is much evidence to show that K^+ may exert an excitatory as well as a depressant action.

14. The effects of low spinal intrathecal injections and of intracisternal and intraventricular injections of KCl and other solutions are compared. In interpreting the latter results, reflex as well as central effects should be taken into account.

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CREATININE LOSSES IN THE SWEAT DURING WORK IN HOT HUMID ENVIRONMENTS

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Except for the work of Talbert and his collaborators (Talbert, Silvers & Johnson, 1926; Talbert & Haugen, 1928), few attempts have been made to correlate the concentration of substances in the sweat with the concentration of the same substances in the plasma. When, therefore, a number of renal creatinine clearances were being done on subjects working in a hot humid environment, it was considered desirable to estimate creatinine not only in the plasma and in the urine but also in the sweat. Talbert, Silvers & Johnson (1926) found a direct relationship between the total non-protein nitrogen of blood and sweat. Was there the same direct relationship for the creatinine contents?

METHODS

Creatinine was determined in serial samples of sweat from two subjects after a dose of creatinine, and on other occasions from the same two subjects, and from two others without the administration of creatinine. Creatinine determinations were also done on saliva collected from the subjects during their exposure to heat, in order to compare the creatinine content of sweat with that of another secretion.

The subjects were all men, between the ages of 34 and 22, and fully acclimatized to work under the environmental conditions of the test. Each exposure was for 160 min. in an air-conditioned chamber maintained at a dry-bulb temperature of 100° F. and a wet-bulb temperature of 93° F. (relative humidity 77 %); the air movement was below 50 ft./min. The men were naked except for a bag completely enclosing one arm in which the sweat was collected.

The complete experimental routine was as follows:

6.00 a.m.	Subject drank 1000 c.c. water.
7.30 a.m.	Breakfast—not standardized.
8.00 a.m.	Subject drank 500 c.c. of 5 % creatinine solution in water if its clearance was being estimated.
8.45 a.m.	Blood sample taken.
9.00 a.m.	Subject entered climatic chamber
11.40 a.m.	Subject left climatic chamber.
11.45 a.m.	Blood sample taken.

The subjects were weighed to the nearest 5 g. immediately on entering the chamber, and their rectal temperatures and standing pulse rates were taken; these three measurements were repeated 10 min. later, the subjects having been sitting quietly meanwhile. The following routine was then carried out for 6 cycles of 25 min. each:

0– 5 min. Rest.	16–24 min. Rest.
5–15 min. Work.	24–25 min. Weigh.
15–16 min. Weigh.	

Sweat samples were taken after each weighing; standing pulse rates and rectal temperatures were taken before and after each bout of work. The work consisted of stepping on and off a stool 1 ft. high; for the first two cycles the rate of stepping was 24 times per min. (metabolic cost 250 kg.cal./hr.) and for the remaining cycles 12 times per min. (metabolic cost 150 kg.cal./hr.); in the fifth cycle, however, only 5 min. work was done, giving the subject an extra 5 min. rest without which he would often have been unable to continue for the sixth cycle; in some cases, when they were not allowed to drink, the subjects were unable to complete six cycles and left the chamber after 110 min. The mean metabolic rate for the full test was 120 kg.cal./hr. In all cases the subjects worked to exhaustion. The water and salt intake of the subjects while in the chamber varied in different tests, according to the other needs of the experiment, but within each experiment it was strictly controlled; in addition the chloride content of each sample of sweat was estimated and as the sweat rate was measured for each cycle it was therefore possible to calculate the exact salt and water balance of the subjects at any time during the exposure. Various conditions were imposed in different exposures, between the gross over-replacement of both salt and water losses and no intake of either.

Sweat was collected in impermeable bags which enclosed the whole of the arm from just below the axilla. The arms were washed first with soap and water, then with distilled water, until the washings were chloride free. The arms were then dried before the dry and chemically clean bags were put on. Once sweating was established samples could be obtained every 10 or 15 min. as required. The sweat collected in this way was only faintly cloudy after it had stood for some time; it was not possible to remove this residual cloudiness.

Blood was obtained by venepuncture, with a minimum of stasis. The samples were collected under medicinal liquid paraffin, with potassium oxalate as the anti-coagulant. Plasma was obtained by centrifuging the blood under paraffin wax.

In certain of the experiments saliva was collected by spitting into bottles; no saliva samples were taken immediately after drinking. All the saliva collected from one subject during one exposure was pooled to make a single sample, which was cleared by filtering through a hard paper. Thus, whereas for each exposure there were a number of samples of sweat, two for each cycle, there was only one sample of saliva.

Creatinine in the sweat and in the saliva was estimated directly; for plasma, the estimation was done on the filtrate after precipitating the protein with the Folin-Wu mixed reagents. The method of Peters & Van Slyke (1932) was used, but, instead of the standard recommended, a stronger one was used consisting of 3 c.c. of creatinine solution, 0·06 mg./c.c., with 17 c.c. of water. The comparisons were made in a photoelectric colorimeter, using monochromatic light, wavelength 500 Å. In all cases therefore only apparent creatinine was being measured. When the sample was not clear a correction for cloudiness was obtained by measuring the absorption of the sample with added reagents immediately on mixing and before the colour had time to develop. The accuracy of this estimation is considerably greater than that of visual colorimetry, being about ±1 %.

RESULTS

The over-all physiological response was substantially the same whatever the salt and water intake; there were some slight quantitative differences which could be correlated with differences in the salt and water balances.

These differences will be discussed in a separate paper; but in all cases the general pattern was as shown in Fig. 1. The rectal temperature rose to about 102° F. in the first 70 min., there was then often a slight fall before a steady value was reached. The standing pulse rate immediately after work rose to about 160 beats/min., and there was only partial recovery during rest. The sweat rate rose rapidly and reached a maximum, in some cases of more than
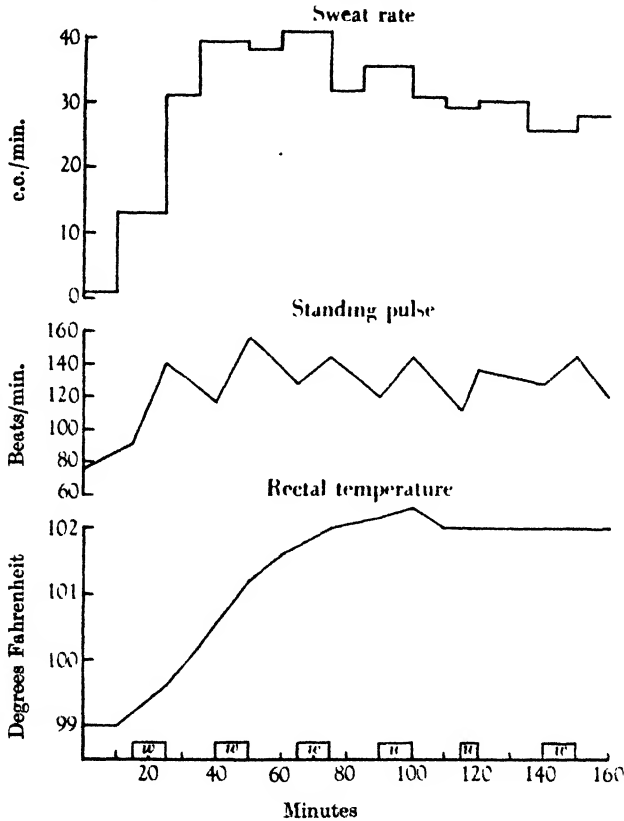
Fig. 1. Sweat rate, pulse rate (standing) and rectal temperature during exposure to heat. Actual results from experiments on subject B. (8 October 1945). Full water and salt replacement. Work periods represented by blocks marked *w*.

60 c.c./min., before the maximum rectal temperature was reached; it then usually showed a steady diminution for the rest of the exposure; this falling off of sweat rate during a long and severe exposure to heat has been described by others, including Robinson, Turrell & Gerking (1945).

In all cases the chloride concentration was relatively low at the beginning of each exposure and rose as the exposure continued; this has been described elsewhere (Ladell, 1945a). In all exposures the creatinine content of the initial sample of sweat was, on the other hand, high, irrespective of whether
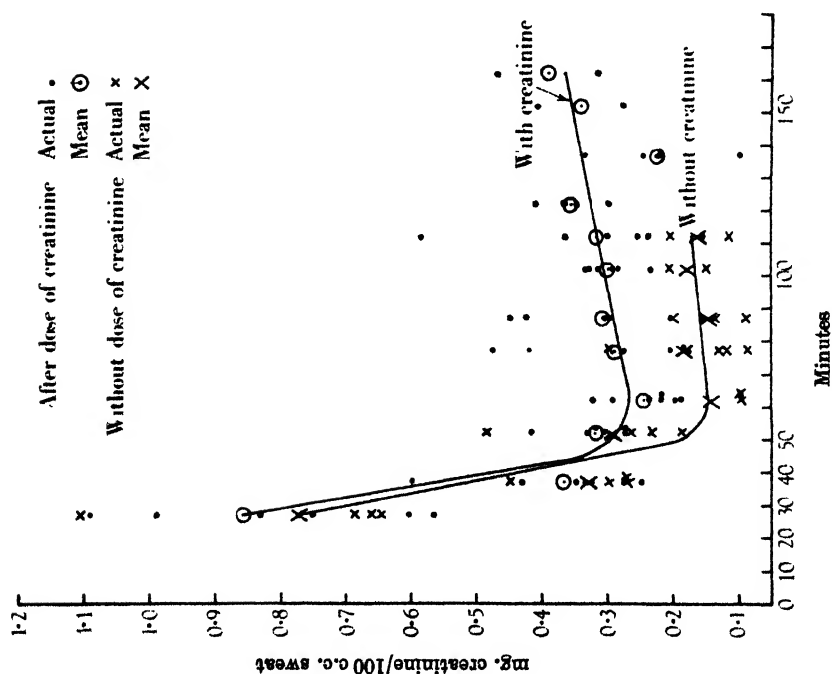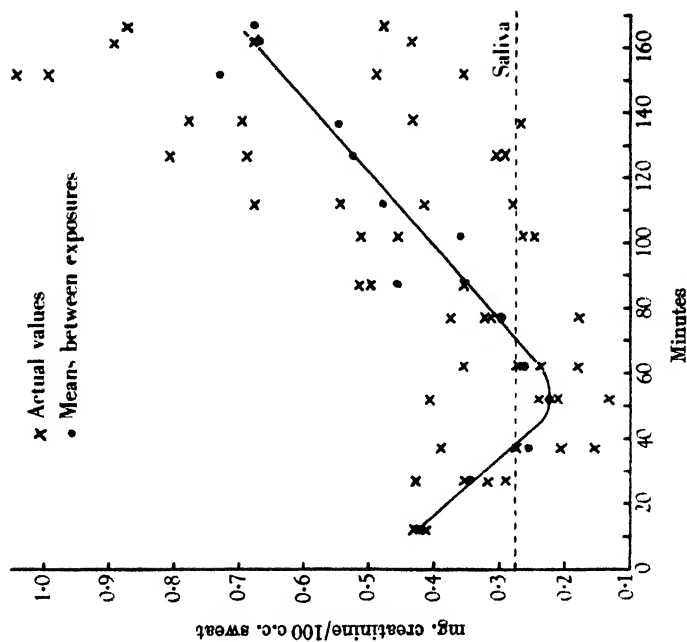
Fig. 2. Changes in creatinine concentration in the sweat during exposure to heat. (a) Subject G.: with and without a dose of creatinine before the test. (b) Subject L.: without creatinine. The dotted line indicates the concentration of creatinine in the saliva (mean of three observations).

the subject had had creatinine of not. High concentrations were sometimes found at the end as well as at the beginning; in the mid-period low values were usually found (see Fig. 2, *a*, *b*). In subjects G. and B. the concentration of creatinine in the early samples of sweat was more than 1·0 mg./100 c.c. In all subjects the concentration fell to 0·3 mg./100 c.c. or less when the sweat rate was greatest. Subject L., who reached a high maximum sweat rate soon after entering the chamber, had the lowest initial concentrations, but the lowest of all the series was found with B., whose sweat on one occasion contained no detectable creatinine.

The effect of a single dose of creatinine before the exposure was followed in subjects G. and B. Ignoring the first samples from each exposure, which were taken before sweating was fully established and in which, as already pointed out, the creatinine contents were uniformly high, the creatinine content of the sweat was 50 % higher after taking creatinine. The results are summarized, with some statistical details, in Table 1; this table also shows, for comparison, the mean values for the two subjects who did not have creatinine.

TABLE 1. Creatinine content of sweat with and without a dose of creatinine before exposure to heat. Values in mg./100 c.c.

	No creatinine			After creatinine			Comparison between means with and without creatinine		
	No. of			No. of			Difference between means	Stand. error of diff. between means	Prob. of difference occurring by chance
Subject	Samples	Ex-posures	Mean	Samples	Ex-posures	Mean			
G.	19	4	0·211	52	7	0·302	0·091	0·029	<0·003
B.	20	3	0·167	14	2	0·253	0·086	0·044	0·05
Results from G. and B. as a single series	39	7	0·189	66	9	0·291	0·102	0·024	0·003
P.	11	1	0·275	—	—	—	—	—	—
L.	49	4	0·439	—	—	—	—	—	—

Note. The first sample in each exposure is not included in the above figures.

Preliminary statistical analysis shows that not much weight can be given to this 50 % difference, since, although the difference is highly significant for subject G., and slightly less so for the results from G. and B. pooled and treated together, it is of doubtful significance for B.

By contrast, the effect of a dose of creatinine on the creatinine content of the saliva was clear. During each of a number of exposures a single sample of saliva was taken. The mean creatinine concentration in seven such samples of saliva from G. and B., when they had no creatinine, was 0·455 mg./100 c.c.; in another seven samples, after they had had creatinine, it was 1·379 mg./100 c.c., a three-fold increase. The standard error of the difference between the means was 0·236; thus the difference of 0·924 mg. is highly significant and may be accepted as due to the higher creatinine content of the blood. Neither P. nor L.

had creatinine; the creatinine content of P.'s saliva, one observation only, was 0·318 mg./100 c.c.; and the mean of three observations on saliva from L. was 0·276 mg./100 c.c. A comparison of these figures with the tables and with Fig. 2 (*b*), shows that, after taking creatinine, the creatinine content of the saliva is higher than that of the sweat, but that without creatinine it tends to be lower. In no case was a low salivary creatinine found when the blood creatinine was raised.

With one exception, the creatinine content of the blood was only measured on the subjects who had had creatinine. The mean of nine estimations on plasma from subjects 45 min. after taking 5 g. creatinine by mouth was 5·20 mg./100 c.c., and of eight estimations on samples obtained when they came out of the heat was 5·92 mg./100 c.c. Table 2, which gives the results in detail,

TABLE 2. *The creatinine of plasma in subjects* G. *and* B. *after* 5 g. *creatinine by mouth, and in* L. *without creatinine*

Subject	Plasma creatinine (mg./100 c.c.)	
	Before entering chamber	After leaving chamber
G.	2·44	—
	5·88	8·32
	5·553	5·422
	6·550	4·908
	4·807	4·871
	4·315	4·633
	6·830	7·450
B.	8·29	5·01
	2·196	6·805
L.	—	0·809

shows that the effect of the exposure to the heat on the creatinine content of the plasma was variable. The normal blood creatinine is stated by Harrison (1944) to lie between 0·7 and 2·0 mg./100 c.c.; hence the 5 g. dose of creatinine had raised the concentration by at least three-fold, as it did that in the saliva.

It was thought that the high creatinine content of the sweat samples obtained at the beginning of each exposure might have been due to substances giving the Jaffe reaction being washed off the skin by the first sweat. Three tests were therefore done on subject G. to investigate this possibility; G. was chosen because he showed the highest initial concentrations. In these tests the subject put a bag on his arm but remained in the cold; several small quantities of warm distilled water were then successively introduced into the bag. This water was rubbed all over the arm through the bag, and after 25 min., when about 30 c.c. of water had been introduced (an amount comparable to the volume of sweat produced from an arm in the first 25 min. exposure to the heat) a sample was taken and its creatinine content estimated. In two of these tests the subject washed his arm thoroughly with tap water and then with distilled water before putting on the bag; in these the apparent creatinine

content was 0·242 mg. and 0·158 mg./100 c.c. respectively; in the third test the arm was not washed, but the creatinine content of the sample was 0·101 mg./100 c.c. As the creatinine content of the initial sweat was much greater than this on all occasions, the presence of substances on the skin surface giving the Jaffe reaction cannot be the cause of the high concentrations; on the other hand, it is not possible to exclude the accumulation of such substances in the lumen of the sweat glands when they are at rest.
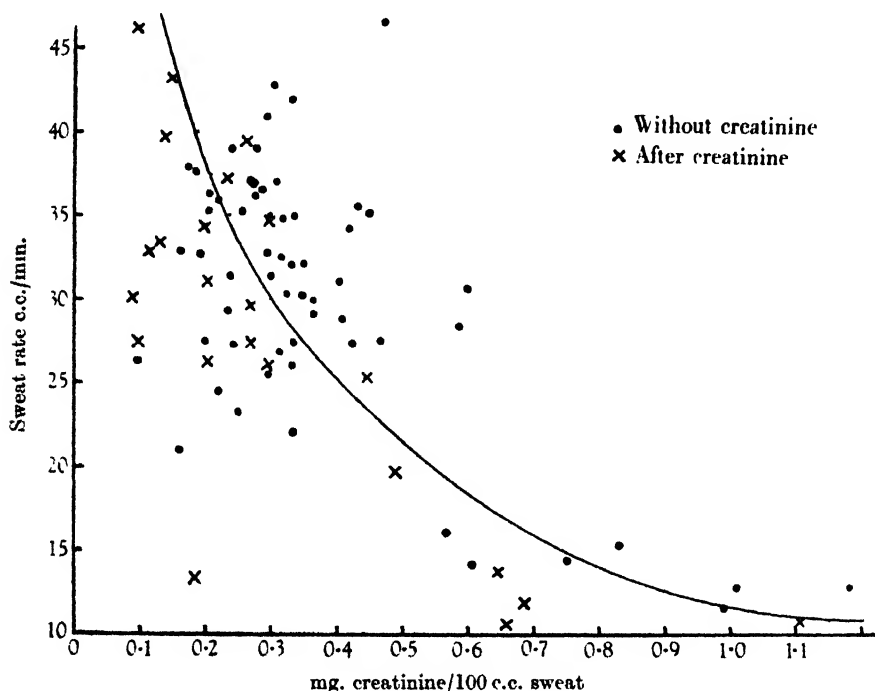
Fig. 3. Subject G. Relation between the creatinine concentration in the sweat and the sweat rate. The curve is the regression line for concentration and the reciprocal of the sweat rate; it has a correlation coefficient of 0·93.

In general, the higher the sweat rates the less creatinine was found in the sweat, and conversely. The subjects who had low initial sweat rates, G. and B., had more creatinine in their early samples than had L. who began to sweat fast almost immediately on entering the chamber. Statistical tests confirmed this impression. For 186 observations there was a correlation coefficient of 0·58 between creatinine concentration in the sweat and the reciprocal of the sweat rate. Taking each of the three subjects G., B. and L. separately, the correlation coefficients were 0·93, 0·72 and 0·39 respectively for 83, 39 and 51 observations; the results for P. were not analysed separately. All these coefficients are significant. In Fig. 3 the sweat rate is plotted against the creatinine concentration for subject G.; the regression line is also shown.

DISCUSSION

Whereas the creatinine contents of both the saliva and the blood increase three-fold after a dose of 5 g. of creatinine, there is only a slight increase in the creatinine content of the sweat. The sweat creatinine appears to be more closely linked with the rate of sweating than with the level of creatinine in the blood; thus the slower the rate of sweating the greater the creatinine content. Statistically there is good correlation between the reciprocal of the sweat rate and the sweat creatinine. This reciprocal relationship between creatinine content and sweat rate is the opposite of what has been observed for chloride concentration (Ladell, 1945b); a number of other authors have also reported that the greater the sweat rate the more nearly does the chloride content of the sweat approach that of the plasma (Dill, Hall & Edwards, 1938; Johnson, Pitts & Consolazio, 1944). There would therefore appear to be two mechanisms at least involved in the production of sweat from body fluids. Mosher's (1933) suggestion that there is an essential similarity between sweat and urine may be correct; but the fact that the creatinine content of the sweat is little affected by that of the blood, whereas the urinary creatinine is greatly affected, indicates that the mode of production of the two fluids must be very different.

SUMMARY

1. The creatinine contents of serial samples of sweat from men working in a hot humid environment were measured and compared with the creatinine contents of saliva and of plasma obtained at the same time.

2. Increasing the creatinine content of the blood raised that of the saliva correspondingly, but had little effect on that of the sweat.

3. There is a reciprocal relationship between sweat rate and the creatinine content of sweat.

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THE RESPONSE OF NEWBORN RATS TO ADMINISTRATION OF WATER BY THE STOMACH

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The antidiuretic hormone content of newborn rat pituitary glands has been shown to be much lower than that of the glands of adult animals (Heller, 1947). This finding, when correlated with morphological evidence for the immaturity of the mammalian neurohypophysis at birth, suggested the possibility of neurohypophysial hypofunction in relation to the kidney of the newborn. If such hypofunction existed, the water metabolism of the newborn would be expected to show certain features which have been observed in cases of diabetes insipidus in the adult. For instance, Klisiecki, Pickford, Rothschild & Verney (1933) observed an earlier onset of diuresis after water administration by mouth when the responses of a normal man were compared with those of a man suffering from diabetes insipidus. The response of newborn rats to water administration was therefore investigated.

METHODS

Experimental animals. 547 male albino rats, 18–42 hr. old (weighing from 4·2 to 6·5 g.) and ninety-three male adult rats were used. The adults, which were of the same strain as the newborn animals, were kept on a standard diet.

The mode of administration of water to newborn rats. Attempts to give the standard dose of water, 4·5 c.c./100 g. rat, by mouth were rarely successful. Intra-abdominal or intramuscular injection led to considerable and protracted losses from the site of the injection. The following method of water administration was finally adopted: the animals were removed from the breeding cages shortly after they had been suckled, i.e. when milk in the stomach could be seen through the abdominal wall. Water was then injected into the stomach through the abdominal wall, the milk content of the stomach serving as a guide for the injection needle. Water warmed to about 35° C. had to be administered to avoid sudden gastric contractions.

A series of control experiments were done to ascertain that water administered in this manner was quantitatively placed into the alimentary tract. A number of newborn rats were weighed on an analytical balance, water injections made as above, and the animals weighed again. The precise amount of water given could thus be determined. It amounted to 4·54 + 0·041 (s.e.) g./100 g. in a series of twelve animals. The rats were then killed by decapitation, cardia and rectum ligatured and the gastro-intestinal tract weighed. The mean difference between the weight of the alimentary

tract of these animals and that of twelve litter controls was 4.58 ± 0.454 g./100 g. showing that the stomach and the intestine contained all the injected water up to 6 min. after the intragastric injection.

The collection of urine in newborn rats. The rats were placed singly into small beakers lined with filter paper. Animals which passed urine during the period of experimentation were discarded. In other series, quantitative collection of urine was ensured by ligature of the penis. Statistical comparison of a number of series showed that the urinary output in the latter animals was the same as in newborn rats whose penis had not been tied ($P > 0.9$).

Control experiments showed that it is not always possible to empty the bladder of newborn rats by suprapubic pressure. To collect the bladder urine quantitatively the animals were, therefore, killed by decapitation and the urine withdrawn by bladder puncture. The quantities thus obtained were so small (ranging approximately from 0.01 to 0.15 g. of urine) that it seemed preferable to weigh the urine rather than to rely on determinations of volume. Moreover, the specific gravity of the urines of newborn rats, collected under various experimental conditions, varied so little that it seemed permissible to equate weight with volume without undue loss of accuracy.

Determinations of extrarenal water loss

Newborn rats. The extrarenal loss was determined by weighing the rats on an analytical balance. Animals which passed faeces or urine were discarded. In later series the penis was tied to avoid loss of urine. Comparison showed that the rate of extrarenal loss was not changed by this procedure ($P > 0.9$).

Adult rats. The animals were put singly into glass metabolism cages fitted with urine/faeces separators. The body weight, the weight of the urine and that of the faeces were determined from hour to hour. The urine was collected under paraffin oil. The air temperature in these as well as in the experiments on newborn rats was controlled thermostatically. No attempt, however, was made to control air humidity.

The choice of air temperature at which experiments on newborn rats were performed

It is not easy to decide at which environmental temperature experiments on newborn rats should be conducted. Full capacity for thermoregulation is only acquired by rats during the fourth week of extra-uterine life (Brody, 1943). For instance, Gullick (1937) showed that rats aged 6 days kept at air temperature ranging from 24 to 37° C. acquired a rectal temperature approximately 1.5° C. higher than that of the surrounding air while the rectal temperature dropped to less than 0.5° C. above that of the air if such animals were exposed to temperatures of 20° C. or lower for 1 hr. Newborn rats derive much of their warmth from the mother, i.e. from the contact with a surface at about 31° C. (Herrington, 1940), and this would suggest that 'physiological' experiments on newborn rats should be performed at about this temperature. However, it should be considered that the mother leaves the young rats for considerable periods (e.g. when feeding) with the result that lower body temperatures are likely to occur in newborn rats. These physiological fluctuations of body temperature can hardly be imitated experimentally and it was therefore decided to conduct experiments at two levels of air temperature. The levels chosen were: (1) the average room temperature ($20/21^\circ$ C.), and (2) that of the approximate skin temperature of adult rats ($30/31^\circ$ C.). The experimental results on newborn rats were therefore obtained at the lowest and at the highest temperatures which these animals were likely to encounter.

<div align="center">RESULTS</div>

<div align="center">*The effect of water administration on the urine flow of newborn rats*</div>

The conduction of diuresis experiments on normal adult rats is much facilitated by the ease with which the animals can be made to void urine at any time chosen by the investigator: prodding and, if necessary, suprapubic abdominal

pressure is sure to induce complete emptying of the bladder. In newborn rats, gentle stroking of the perineal region, aided by digital pressure on the abdomen, frequently induced voiding of considerable volumes of urine, but control experiments showed that this procedure led to complete emptying of the bladder in a minority of newborn animals only. A series of twenty-five rats, weighing from 4·85 to 6·14 g., were made to deliver as much urine as possible by suprapubic pressure, the animals were then decapitated, and residual urine withdrawn by direct puncture of the bladder. The figures for the mean weight of residual urine of the series were $0·0293 \pm 0·00532$ (s.e.) g. or $0·52 \pm 0·091$ g./100 g. In view of these results the following procedure was adopted to show the rate of water diuresis in newborn rats. The bladder of a number of male litter mates was 'emptied' by suprapubic pressure. The animals were then divided into two series. One series received about 4·5 c.c. water/100 g. rat by injection into the stomach; the second series received no water. After a given time the animals in both series were killed and the urine withdrawn by bladder puncture. The urines were weighed singly in tared weighing bottles. A significant difference between the mean urinary weights of the two series would thus be due to the renal excretion of the administered water. However, it will be seen from Fig. 1 A, which shows the results of experiments conducted at 30/31° C., that there was no significant difference between the injected and the control series even when the period allowed for renal excretion of the injected water was extended to 5 hr. The mean weight of the urine in a series of eleven newborn rats killed 1 hr. after water administration was $0·96 \pm 0·073$ (s.e.) g./100 g., that of the control series (eleven animals) was $1·01 \pm 0·092$ g./100 g., the figures for animals killed after 2 hr. were $1·12 \pm 0·151$ g./100 g. (twelve injected animals) and $1·17 \pm 0·142$ g./100 g. (eleven controls), those for animals killed after 3 hr. were $1·68 \pm 0·115$ g./100 g. (twenty-two injected animals) and $1·81 \pm 0·088$ g./100 g. (twenty-three controls) and those for rats killed after 5 hr. $2·16 \pm 0·114$ g./100 g. (fourteen injected animals) and $2·13 \pm 0·114$ g./100 g. (fourteen controls). It should be pointed out that the figures for weight of urine given have to be corrected by deduction of the 'residual urine' (0·52 g./100 g. rat) to represent the urinary output during the periods stated (Fig. 1 A).

No evidence could be obtained from these experiments for the occurrence of water diuresis in newborn rats. Experiments done at 20/21° C. air temperature showed further that the absence of water diuresis was not due to 'heat inhibition' (Heller & Smirk, 1932*b*; Weiner, 1944) at the higher temperature level: the mean amount of urine produced by twenty-one newborn rats injected with water was $1·03 \pm 0·078$ g./100 g. after 4 hr. at 20/21° C.; that of the control series (twenty-two animals) was $1·04 \pm 0·122$ g./100 g.

Fig. 1 B shows the renal response of adult rats which received the same relative amount of water (4·5 c.c./100 g. rat). Control experiments had shown

that the gastro-intestinal tract of the newborn rats contained approximately 3 % of their body weight of milk at the outset of the diuresis experiments (2·71 ± 0·318 g./100 g. rat in a series of twenty animals). The experiments on adult rats were therefore conducted as follows: the animals were starved for 24 hr. but received water *ad libitum*; 3 c.c. cows' milk/100 g. rat was then given by mouth and 4·5 c.c. water/100 g. 10 min. later. Fig. 1 B shows that water diuresis proceeded freely in these animals.
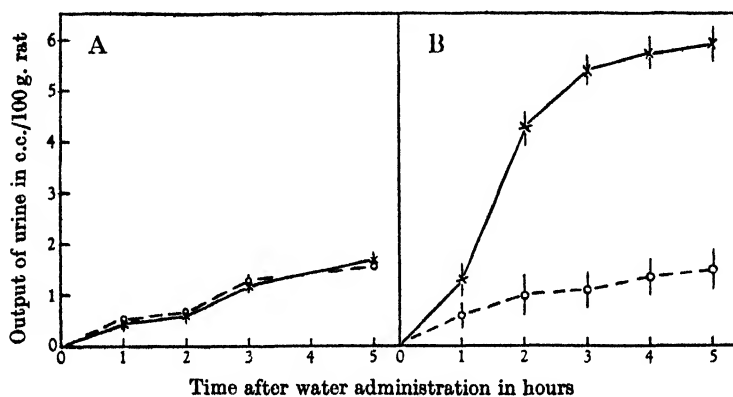
Fig. 1. Comparison between the urinary output of newborn and of adult rats after administration of water by the stomach.

A. ×——× = mean urinary output of newborn rats after the intragastric injection of 4·5 c.c. water/100 g. rat. ○---○ = controls. The figures for the urine volume at various times were obtained by deducting the mean residual urine volume (= mean volume of urine contained in the bladder at the outset of the experiment) from the volume of urine obtained at a given time.

B. ×——× = mean urinary output of adult rats which received 3 c.c. milk/100 g. by mouth and 4·5 c.c. water/100 g. 10 min. later (3 c.c. milk/100 g. rat = approximate content of milk in alimentary tract of newborn rats at outset of diuresis experiments). ○---○ = mean urinary output of adult rats which received 3 c.c. milk/100 g. only. The vertical lines indicate the standard error. There is no evidence that the newborn rats excreted any of the administered water during the 5 hr. of observation.

Further diuresis experiments on adult rats receiving a preparation of 'full cream dried cows' milk,' partly reconstituted to make the concentrations of proteins and fat approximately equal to those of rat milk, gave essentially the same results.

The rate of gastro-intestinal absorption of water in newborn rats

Little seems to be known about the rate of alimentary water absorption in newborn mammals. This factor was, therefore, investigated. The method used to determine the rate of gastro-intestinal water absorption was essentially the same as that used by Heller & Smirk (1932a) in adult rats. The weights of the alimentary tracts of series of rats, killed at given times after the administration of a standard dose of water by stomach tube, were compared with the mean gastro-intestinal weights of a control series. However, the technique of intragastric injection of water into newborn rats made it necessary to modify this simple procedure. It will be remembered that, for intragastric injections, animals had to be used whose stomachs were not empty. A decrease in the weight of the alimentary tract of the injected animals after a given time would therefore

be due not only to the absorption of the injected water but also to the absorption of an unknown quantity of milk. To overcome this difficulty the experiments on newborn rats were conducted as follows. A number of newborn animals (litter mates) were weighed and divided into two series. The first animal was injected with the standard dose of water and the time of injection noted. An animal of the other series was then set aside as a control. The injected animal was weighed to determine the extra water load accurately. This procedure was followed until each of the series of injected rats was provided with a control. After a given time the animals were killed and their alimentary tracts weighed on an analytical balance. The difference between the mean weight of the alimentary tracts of the injected rats (in terms of g./100 g. rat) and that of the
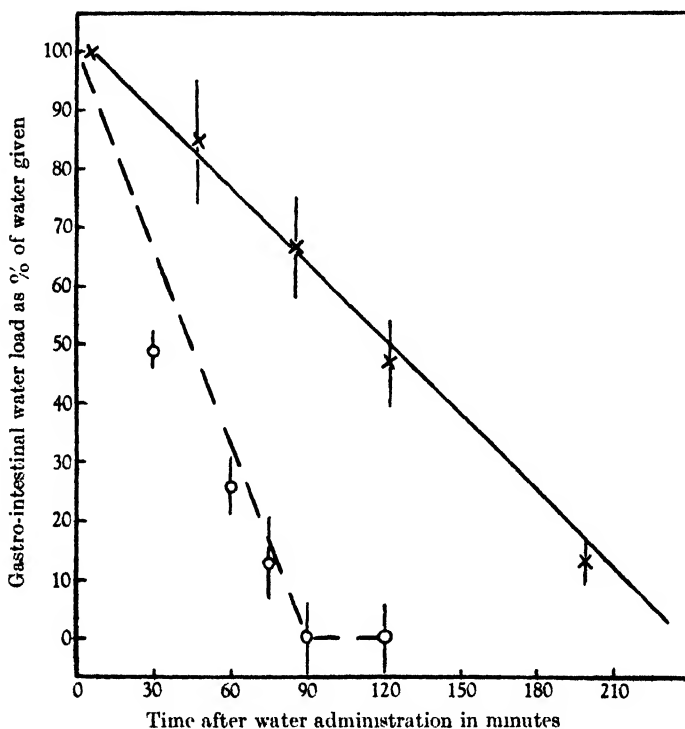
Fig. 2. The rate of alimentary water absorption in newborn (x —— x) and in adult (O – – – O) rats. Both series received 4·5 c.c. water/100 g., the adult rats, in addition, received 3 c.c. milk/ 100 g. 10 min. before the water was administered. The newborn rats were kept at 30/31° C. air temperature, the adult rats at 20/21° C. For results on newborn rats kept at 20/21° C., see text. The slower rate of water absorption in newborn rats is evident.

alimentary tracts of the control series indicated the fraction of the administered water not absorbed. Estimations of urinary weight were not required in these series and the penis was therefore not ligatured. However, two additional series of experiments showed that the rate of intestinal water absorption in newborn rats was not appreciably changed by this procedure.

Experiments on adult rats were conducted in the same manner as in newborn rats except that food (but not water) was withheld for 24 hr. and that the animals received 3 c.c. milk/100 g. 10 min. before 4·5 c.c. water/100 g. was administered.

Fig. 2 shows clearly that the newborn rats absorbed water at a considerably lower rate than adult animals. For instance, 90 min. after the administration

of water, when absorption in the adults was complete, morè than 60 % of the
water given was still in the alimentary tract of a series of newborn animals
kept at 30/31° C. The rate of gastro-intestinal water absorption in newborn
rats was also studied in animals kept at 20/21° C. A statistical comparison
of the figures for the mean alimentary water load 48 and 90 min. after water
had been given with those obtained at the corresponding times in animals
kept at 30/31° C., showed that the rate of water absorption was approximately
the same at both temperatures (t after 48 min. $= 0.263$, $P > 0.7$; t after
90 min. $= 0.261$, $P > 0.7$) indicating that the body temperature, and therefore
the metabolic rate, had little or no influence on the gastro-intestinal absorption
of water.

The rate of extrarenal water loss of newborn rats

It is known (Heller & Smirk, 1932a) that water diuresis in the adult rat,
and in adults of other mammalian species (Klisiecki *et al.* 1933), begins only
when intestinal water absorption is almost complete. The extrarenal water
loss during this period of latency diminishes the volume of extra water
available for renal excretion but the short duration of the latency period in
adult rats makes this decrease comparatively negligible (Heller & Smirk,
1932a). It seemed likely that these relationships would not be the same in
newborn rats. First, it had been shown that alimentary water absorption
proceeded at a much lower rate than in adults (the absorption of the standard
dose of water taking 180–210 instead of approximately 90 min.) and secondly
that no sizable part of the extra water load was excreted by the kidneys up to
5 hr. after administration. The question arose therefore whether the extrarenal
water loss during the long period of alimentary absorption was of such
magnitude that it accounted for the absence of water diuresis in newborn
rats. In other words, did the rate of extrarenal water loss in the newborn
animals keep pace with the rate of alimentary water absorption with the
result that the extra-alimentary water load became negligible or, at any rate,
insufficient to sustain a water diuresis?

Determinations of the rate of extrarenal water loss of newborn and of adult
rats at two levels of air temperature gave the following results (means with
their standard errors):

Extrarenal water loss:

Adult rats at 20/21° C. $= 0.448 \pm 0.0334$ (46) g./100 g./hr.
at 30/31° C. $= 0.459 \pm 0.0327$ (46) g./100 g./hr.

Newborn rats at 20/21° C. $= 0.072 \pm 0.0011$ (43) g./100 g./hr.
at 30/31° C. $= 0.380 \pm 0.0213$ (42) g./100 g./hr.

It is clear from these results, and from the data shown in Fig. 3, that, at the
lower temperature level, the extrarenal water loss of the newborn animals
was considerably lower than that of the adults. The extrarenal water loss of

newborn rats increased very significantly when the temperature was raised to 30/31° C., but this increased rate of water loss did not exceed that of adult animals at 20/21° C., i.e. at room temperature; in fact statistical analysis showed that the two rates were of the same order of magnitude ($t = 1·67$, $P > 0·09$). The extrarenal water loss of newborn rats kept at 30/31° C. was, if anything, lower than that of adult rats kept at approximately the same temperature ($t = 2·02$, $P < 0·05$).
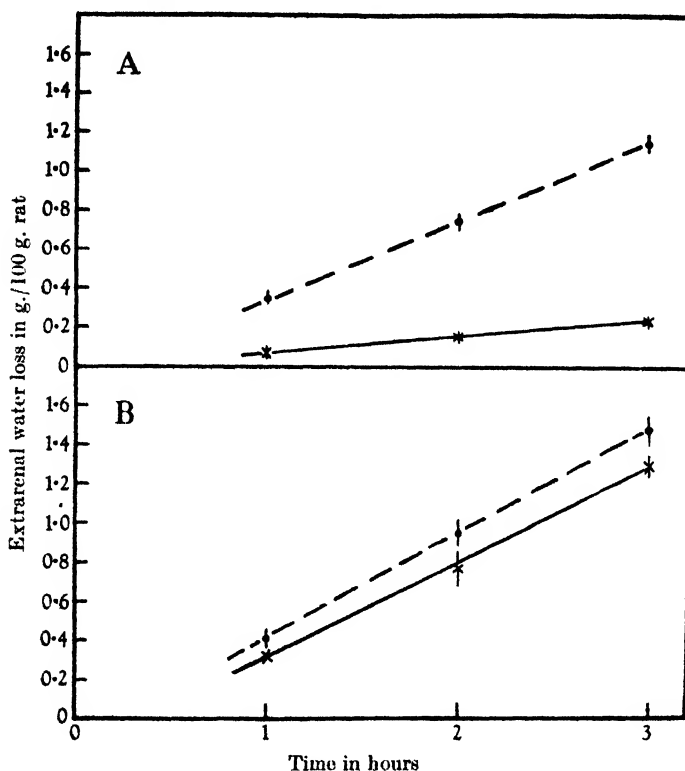
Fig. 3. Extrarenal water loss of newborn (A) and of adult (B) rats at two levels of air temperature.
×——× = extrarenal water loss at 20/21° C. ●---● = extrarenal water loss at 30/31° C.
Results fully explained in text.

The striking difference between the rates of extrarenal water loss of newborn rats at different levels of air temperature is in agreement with the observations of Gullick (1937) and Brody (1943) who showed that rats during the first four weeks of extra-uterine life are not completely homoiothermal. These authors found that, in such animals, body temperature and basal metabolism were closely correlated with the air temperature.

It is evident from the reported data that the *rate* of extrarenal water loss of newborn rats was not higher than that of adults. However, it still remains to be shown how the protracted course of the alimentary water absorption

influences the tissue water load. In other words, given a rate of extrarenal water loss equal or lower than that of adults, what is the tissue water load of newborn rats at the time when the gastro-intestinal absorption of the administered water has been completed?

The following experiments were made to estimate this quantity which will be called the 'residual water load'. The bladder of a number of newborn rats was emptied, the penis ligatured and the body weight determined. Water was then administered by intragastric injection and the exact amount given (= initial water load) ascertained by reweighing. After a given time the rats were weighed again to estimate the extrarenal water loss, killed immediately after the last weighing, the bladder urine withdrawn and its weight determined. An equal number of non-injected controls were treated in an identical manner. It will be seen from Table 1 that there was no significant difference

TABLE 1. Estimation of 'residual' (= tissue) water load in newborn rats
after administration of water by the stomach

Duration of experiment (= hr. after water administration)	Air temp. in ° C.	No. of rats used	Amount of water administered (= initial water load) in g./100 g. rat	Extrarenal water loss during experiment in g./100 g.	Weight of bladder urine at end of experiment in g./100 g. (in brackets): weight of urine of control series (residual urine not deducted)	Difference between weight of urine in animals with extra water and controls (Fisher's t test)	Residual water load in % of initial water load
3	30/31	22	4·50 ± 0·080	1·43 ± 0·080	1·68 ± 0·115 (1·81 ± 0·088)	t = 0·09 P > 0·9	67·4 ± 1·86
5	30/31	19	4·70 ± 0·105	1·61 ± 0·106	2·16 ± 0·114 (2·13 ± 0 114)	t = 0·01 P > 0·9	62·8 ± 2·31
4	20/21	21	4·47 ± 0·083	0·41 ± 0·037	1·03 ± 0·078 (1·01 ± 0·122)	t = 0·07 P > 0·9	90·9 ± 0·86

Since gastro-intestinal absorption of the dose of water given (4·5 c.c./100 g. rat) was completed after about 3 hr. (see Fig. 2) and since the newborn rats did not excrete the extra water by the renal route (see the present table), the tissue water load at any time after completion of gastro-intestinal water absorption was equal to the 'initial water load' minus the extrarenal water loss. Results are given as means with their standard errors.

between the mean urine volumes in any of the three series of experiments performed. That is to say, none of the administered water had been excreted by the kidneys. The residual water load could therefore be calculated by deducting the extrarenal water loss from the initial water load. Table 1 shows that the mean residual water load in a series of rats kept at 30/31° C. was still about 70 % of the initial water load 3 hr. after water had been administered, i.e. at a time when alimentary water absorption had been completed. It is thus clear that it was not the extrarenal water loss which prevented a water diuresis in the newborn rats. Moreover, the high figures for the residual water load after 4 hr. at 20/21° C. and after 5 hr. at 30/31° C. showed (Table 1) that newborn rats were unable to excrete 'extra water' by the kidney even when comparatively large, amounts of it were demonstrably lodged in the extra-alimentary tissues.

DISCUSSION

Evidence is accumulating that the water metabolism of newborn infants and animals differs in many points from that of the adults of the species. This could probably be expected from the very different cell milieu of newborn mammals but recent work indicates that these differences extend to the function of the kidney (McCance & Young, 1941; Heller, 1944) and that of integrative organs like the posterior pituitary gland (Heller, 1947).

The present investigation on the water metabolism of newborn rats is less concerned with any single functional factor than with the complex phenomenon of 'water diuresis'. The results show clearly that water diuresis, as found in adult animals, does not occur in newborn rats. No evidence could be obtained that a dose of water, placed in the alimentary tract of normally hydrated newborn rats, increased the urinary volume during 5 hr. after water administration. Experiments on infants (Lasch, 1923) and on young dogs (Adolph, 1943) indicate that an incompletely developed ability to reduce an excess water load by the renal route is not confined to newborn rats but occurs, though probably to a lesser degree, in other mammalian species.

Of the many factors which are known to influence the course of a water diuresis in adult animals, two only have been investigated in the present work. It was found that newborn rats absorbed water from the gastro-intestinal tract at a lower rate than adult animals of the same strain. This delay in the alimentary water absorption would by itself lead to a 'flattening' of the curve of water diuresis. However, it could be shown that this is not the only or even the most important factor which modifies water diuresis in newborn rats. (This seemed to be of importance since it is not known whether a similar retardation of alimentary water absorption occurs in other mammalian species. The relative immaturity of newborn rats will be remembered in this connexion.) Further investigation indicated that newborn rats were unable to excrete 'extra water' at the adult rate even after alimentary absorption had been completed. Determinations of the rate of extrarenal water loss of newborn rats—the second factor investigated—revealed that the rate of loss did not exceed that of adults at similar air temperature and showed also that, during the protracted period of alimentary water absorption, only a fraction of the initial water load was lost by the extrarenal route. Alimentary water absorption of the standard dose of water in newborn rats was shown to be completed in about 3 hr., and since it had also been shown that the urine volume did not increase during this time, it follows that any of the administered water residual after this period was lodged in the extra-alimentary tissues. In a series of rats kept at 30/31° C., it could be shown, for instance, that the mean residual (= tissue) water load after 3 hr. amounted to about 70 % of the dose of water administered and to about 60 % 5 hr. after the water had been given.

The latter finding would seem to have some bearing on a clinical problem. It is well known that infants within 2 or 3 days of birth are liable to develop a condition called 'oedema of the newborn' (Hallum, 1941). The incidence of this condition, which in Hallum's series progressed in some cases to generalized anasarca, was low (1·2 %) in children born at or near full term but comparatively high (13·4 %) in infants with a maturity of less than 36 weeks. It will also be remembered that oedema after parenteral fluid administration is frequently observed in newborn infants. It has been shown that newborn rats failed to give a diuretic response to water and that this failure produced a rise of the body water content which lasted for many hours. In other words, a dose of water which, owing to the quick alimentary absorption and quick renal excretion, increases the tissue water load of adult rats slightly and for a short period only (Heller & Smirk, 1932a) led to a state of 'oedema' in normal newborn rats. A comparison between the response of adult human beings and infants to the same relative extra water load may show whether the same factors contribute to the occurrence of oedema in newborn children.

SUMMARY

1. No evidence for an increase of urinary output could be obtained when a dose of water was placed in the alimentary tract of normally hydrated newborn rats and the urinary output measured for the following 5 hr.

2. The diuretic response to water (=water diuresis) is either very much diminished or completely absent in newborn rats.

3. The rate of gastro-intestinal absorption of water in newborn rats was estimated at two levels of air temperature (20/21 and 30/31° C.) and was found to be lower than in adult animals.

4. The body temperature, and therefore the metabolic rate, had little or no influence on the gastro-intestinal absorption of water in newborn rats.

5. The extrarenal water loss, per unit body weight, of newborn rats kept at 20/21° C. was only a small fraction of that of adult animals kept at the same air temperature. Newborn rats kept at 30/31° C. lost water extrarenally at about the same rate as adult rats at 20/21° C.

6. It could be shown that the slow alimentary absorption of water was not the only factor which prevented water diuresis in newborn rats. Newborn animals were unable to reduce an extra water load by renal excretion even when comparatively large amounts of water (about 70 % of the water administered) were demonstrably lodged in the extra-alimentary tissues.

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THE RESPONSE OF ADULT AND SUCKLING RATS TO THE ADMINISTRATION OF WATER AND OF HYPERTONIC SOLUTIONS OF UREA AND SALT

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The work which has been done in the last few years has emphasized the fact that hypertonic solutions of chlorides and of urea provoke a large diuresis in dehydrated subjects (Adolph, 1921; Hervey, McCance & Tayler, 1946a, b). Most of the work in this country has been done on adult human subjects and in America on adult dogs (Elkinton & Taffel, 1942), but there is evidence in the literature that, for some time after birth, the kidney may not function as it does in adult life. The work now to be reported was undertaken (a) to study the effects of hypertonic solutions upon dehydrated adult animals, (b) to compare the effects of treating newborn animals in a similar way. In connexion with this the responses of adult and suckling animals to comparable amounts of water have also been investigated. Work on animals other than rats will be reported later.

METHODS

Animals. In the experiments on adult animals the rats used were of both sexes. They were 6 months old; the females weighed about 250 g. and the males about 350 g. Throughout the experiments they were maintained on a standard stock diet. The young animals were taken away from their mothers as soon as the last member of the litter had been born. They were unfed. Throughout the experiment they were kept in a box lined with cotton-wool and warmed with an electric bulb to about 30° C. Ten or more were used for each experiment.

Urine collections. Food and water were removed from the cages of the adult rats 18 hr. before the beginning of an experiment. The bladders of the animals were emptied before and at approximately hourly intervals during each experiment. This was done by gently compressing the abdomen, and, with practice, it was possible to feel how full their bladders were and when they were empty. The rats were kept during the experiments in aluminium metabolism cages resting on glass funnels, and the urine was collected under toluene in finely graduated cylinders of 10 c.c. capacity. At the end of each collecting period the urine expressed manually was added to the amount which had collected in the cylinder.

The urine from the newborn rats was collected in small weighed bottles. At the beginning of the experiment the bladders were emptied by compression and gentle stroking of the perineum. It seems that in the early stages of life these animals do not empty their bladders spontaneously, but depend upon a reflex initiated by the mothers licking the perineum. The emptying of the bladders was repeated at the end of each collecting period.

Administration of solutions. The adult rats received all solutions by stomach tube. The finest commercial rubber tubing was too thick to pass into the stomach of the newborn rats, but it was possible to remove the insulation tubing from the finest electrical wire, and to pass this down into the stomach of the sucklings. Most solutions were given by this means, but some were administered intraperitoneally to compare the degree of absorption from the intestine and the peritoneal cavity. Both adult and suckling animals received comparable amounts of the solutions. Usually 5% of the body weight was given.

Collection of blood. Two samples of blood were taken in each experiment. Blood was obtained from the tips of the tails of the adult animals and collected in heparinized tubes under paraffin. It was centrifuged immediately and the plasma separated. The first sample was taken in the middle of the preliminary (control) period, before the experimental solution had been administered, and the second in the middle of the period during which the peak of the diuresis was expected to fall. Blood was obtained from the newborn animals by snipping off their heads. As a result, the samples of blood had to be taken at the ends of the control and experimental periods. Half the newborn rats in each experiment were, therefore, killed at the end of the preliminary collection of urine, and the remainder at the end of the experiment. The blood was collected under paraffin in heparinized 'Durham' tubes which were centrifuged at once to separate the plasma.

Chemical estimations. Urea was estimated in blood and urine by the xanthydrol method (Lee & Widdowson, 1937). Chlorides were estimated by the ultramicro method of Sendroy (1937). When enough urine was available, chlorides were also estimated as described by McCance (1937). The depression of the freezing point was determined either by a Beckmann thermometer or by a micromethod which required less than 0·1 c.c. urine. The difference in potential between thermocouples in the unknown solution at its freezing point and in melting ice made from distilled water was measured by means of a sensitive galvanometer and potentiometer. The freezing points of a series of standard solutions were determined by means of a Beckmann thermometer and from these a graph was plotted relating freezing point to the reading on the galvanometer scale. In the tables the results have been expressed in m.osmol./l. by calculation from the observed freezing point.

RESULTS

The response of adult and infant rats to hypertonic solutions of NaCl

The response of adult rats to hypertonic solutions of salt seems to be of the same nature as the response of human adults. The data of a typical experiment are given in Table 1. The most obvious feature of this response has been the very great diuresis. It will be observed that the volume of urine passed by rats which had been dehydrated for 18 hr. was very low, and that it had a very

TABLE 1. The response of adult rats, which had been kept without water for 18 hr., to a hypertonic solution of sodium chloride given by stomach tube

Time	Minute volume c.c./1000 g. rat	Urine			Blood chloride m.equiv./l.
		Osmotic pressure m.osmol./l.	Chloride m.equiv./l.	Urea m.mol./l.	
9.45–11.45 a.m.	0·003	2560	290	1220	106
11.45 a.m. 5% of the body weight 10% NaCl given					
12.0 noon–1.0 p.m.	0·069	1620	416	720	—
1.0–2.10 p.m.	0·168	1380	460	340	—
2.10–3.35 p.m.	0·198	1310	460	230	—
3.35–4.35 p.m.	0·155	1390	510	230	—
4.0 p.m. Blood	—	—	—	—	116
4.35–5.35 p.m.	0·088	1440	538	235	—
5.35–7.15 p.m.	—	1530	560	560	—

high osmotic pressure. During the first hour after the administration of the NaCl solution the minute volume increased by more than twenty times and continued to rise for the next 2 hr. Later the minute volume fell gradually, but, by the next morning, it was still higher than it had been before the experiment began, and this in spite of the progressive dehydration of the body.

The osmotic pressure of the urine always fell till the diuresis reached its peak, and then gradually rose again, but, even after 12 hr., it had not reached the level of the preliminary sample. The results suggest that, under these conditions, the osmotic pressure of the urine depends upon the minute volume (Hervey, McCance & Tayler, 1946 a, b). The changes in the freezing point were due mainly to changes in the urea concentrations. With the onset of the diuresis the percentage of urea in the urine fell and by the next morning it had not regained its former level. The chloride concentration, on the other hand, rose throughout the experiment and was even higher the next morning. The clearances of both substances were greatest at the peak of the diuresis.

TABLE 2. The response of newborn rats to a hypertonic sodium
chloride solution given by stomach tube

| | Minute volume c.c/1000 g. rat | Urine | | | Blood chloride m.equiv./l. |
Time		Osmotic pressure m.osmol/l.	Chloride m.equiv./l.	Urea m.mol./l.	
12.15–3.15 p.m.	0·035	505	101	133	108
3.15 p.m. 5% of the body weight of 10% NaCl given					
3.15–7.15 p.m.	0·067	623	155	98	—
7.20 p.m. Blood collected					130

The response of the newborn kidney to the administration of salt was very different from that of the adult. Ninety-two newborn rats were used for these experiments. The results of a typical experiment are shown in Table 2. It will be seen that the preliminary urine from the infant rats was much more dilute than the corresponding sample from the adult animals. This is an observation well authenticated in man (McCance & Young, 1941; Heller, 1944). A flow of urine with an almost fixed freezing point, irrespective of the degree of hydration of the young rat, seems to be characteristic of the early life of this animal, but this will be considered more fully in connexion with the response of newborn rats to water. After the administration of hypertonic salt solutions, either by intraperitoneal injection or by stomach tube, the blood chloride rose to much higher levels than it did in adults. Both methods of administering the chloride solution caused a very similar increase in blood chloride concentration but, in spite of it, there was no sudden diuresis as in the adult animals. Sometimes the minute volume rose slightly, but, as a rule, there was no significant change. The osmotic pressure of the urine also changed little although the alteration was in the nature of an increase and not of a decrease as in adults. Analyses of the urine for chloride and urea showed how this was brought about. The

chloride concentration rose after the administration of NaCl. The urea concentration fell to a lesser degree and hence there was only a small increase in total osmotic concentration.

The response of adult and infant rats to hypertonic solutions of urea

Adult rats responded to an increased concentration of urea in the body fluids in much the same way as human adults have been shown to do (McCance & Young, 1944), and the response was similar to that seen after the administration of hypertonic NaCl solution (Table 3). A very large diuresis was induced

TABLE 3. The response of adult rats which had been kept without water for 18 hr. to the administration of a hypertonic urea solution by stomach tube

Time	Minute volume c.c./1000 g. rat	Urine Osmotic pressure m.osmol./l.	Chloride m.equiv./l.	Urea m.mol./l.	Blood urea m.mol./l.
10.0 a.m.–12.0 noon	0·003	3000	350	1240	6·6
12.30 p.m. 5% of the body weight 20% urea given					
12.30–1.0 p.m.	0·122	1360	125	750	—
1.0–2.0 p.m.	0·138	1410	105	1050	—
2.0–3.0 p.m.	0·138	1430	88	1060	—
2.30 p.m. Blood collected					40
3.0–4.6 p.m.	0·139	1460	62	1160	—
4.6–5.4 p.m.	0·111	1500	55	1175	— -
5.4–6.0 p.m.	0·098	1580	38	1190	—
6.0–10.0 p.m.	0·017	2560	31	2160	—

by strong urea solutions given by stomach tube, and the urine produced during this diuresis was much more dilute than the preliminary urine. A difference from the response to NaCl solution lay in the fact that, in spite of the rising blood urea, there was a fall in the urea concentration of the urine as the diuresis developed. The chloride concentration fell and remained low. About seventy newborn rats were used for the urea experiments. The newborn rats responded to strong solutions of urea much as they did to those of hypertonic salt solutions (Table 4) and hence both these solutions demonstrate similar differences in behaviour between the kidneys of adults and of newborn animals. The minute volume was increased by a very small amount and the osmotic pressure rose slightly. The concentration of urea in the urine increased nearly

TABLE 4. The response of newborn rats to the administration of hypertonic urea solution by stomach tube

Time	Minute volume c.c./1000 g. rat	Urine Osmotic pressure m.osmol./l.	Chloride m.equiv./l.	Urea m.mol./l.	Blood urea m.mol./l.
9.15 a.m.–12.15 p.m.	0·032	457	81	122	13
12.20 p.m. Blood collected and 5% of the body weight of 20% urea given					
1.0–5.30 p.m.	0·052	483	36	360	88
5.35 p.m. Blood collected					

three times, but the chloride concentration fell so that the total osmotic pressure of the urine did not rise very much. It is to be noted that there is very little variation in the volume or total concentration of the urine of the newborn animals in spite of the great increase in the urea in the blood.

Response of adult and newborn rats to water

The failure of hypertonic solutions of both salt and of urea to provoke a diuresis in newborn rats suggested an investigation of their response to administration of water. The adult response to water was the one to be expected from all previous work on water diuresis. Administration of water by mouth to adult rats caused a rapid rise in minute volume, while the osmotic pressure of the urine fell to a correspondingly low level. The concentrations of chloride and of urea in these urines became, naturally, very low. Experiments were performed on fully hydrated rats as well as on those that had been deprived of water for 18 hr. It was found that this short period of dehydration made no fundamental difference to the response of adult rats to 5 % of their body weight of water. Both series of animals produced a large volume of much more dilute urine. The figures of a typical experiment are given in Table 5. When newborn rats were given a comparable amount of water their response was very different. It can be seen from Table 5 that the introduction of this

TABLE 5. Response of fully hydrated adult rats and of newborn rats to 5 % of their body weight of water given by stomach tube

Time	Minute volume c.c./1000 g. rat	Osmotic pressure of urine m.osmol./l.	Concentration of urea in urine m.mol./l.
	Adult rats		
10.5 a.m.–12.30 p.m.	0·0086	2980	1230
12.30 p.m. Water given			
12.30–1.50 p.m.	0·071	414	174
1.50–2.45 p.m.	0·188	206	84
2.45–4.45 p.m.	0·031	1060	648
	Newborn rats		
10.10 a.m.–12.45 p.m.	0·024	510	375
12.45 p.m. Water given			
1.0–3.0 p.m.	0·025	489	333
3.0–5.0 p.m.	0·032	419	292

volume of water caused no rapid diuresis in these animals in the time taken by the adults to get rid of their excess of water. The minute volume increased only very gradually and the change in the osmotic pressure of the urine was correspondingly small. A comparison of the concentrations of urea in the urines of adult and of newborn animals emphasizes the fixed composition of the urines of these newborn rats.

It was clear from these experiments that there must be a fundamental difference in the response of adult and of newborn rats to water, and that at some stage in the young animal's development a change in function must take

place. The young rats were not injured by these experiments and could be returned to the mother at the end of the day, so some of the litters were investigated again at 4, 8 and 12 days of age. Some of the results are given in Tables 6 and 7. It will be seen that by the time the animals were four days old

TABLE 6. Response of 4-day-old rats to water given by stomach tube

| | | Urine | |
| | | | |
Time	Minute volume c.c./1000 g. rat	Osmotic pressure m.osmol./l.	Urea m.mol./l.
9.40 a.m.-12.40 p.m.	0·027	590	164
12.0 noon-12.20 p.m. 5% of the body weight of water given			
12.20-2.0 p.m.	0·107	310	132
2.0-3.30 p.m.	0·086	242	104
3.30-5.15 p.m.	0·029	565	190

TABLE 7. Response of 12-day-old rats to water given by stomach tube

| | | Urine | |
| | | | |
Time	Minute volume c.c./1000 g. rat	Osmotic pressure m.osmol./l.	Urea m.mol./l.
10.0 a.m.-12 noon	0·049	600	230
12.0 noon 5% of the body weight of water given			
12.0 noon-1.0 p.m.	0·138	452	99
1.0-2.0 p.m.	0·197	188	33
2.0-4.25 p.m.	0·051	520	177

they were beginning to respond to the dose of water in adult fashion by a small diuresis and by a small fall in the osmotic pressure of the urine. At 4 days old, however, the osmotic pressures show that rats still produce a dilute urine and are probably unable to concentrate it as much as an adult can. At 12 days of age the response to water was almost indistinguishable from that of the adult animal but the urine was still dilute in the preliminary sample and at the end of the experiment it returned to this state. This consistently dilute urine is typical of suckling animals.

DISCUSSION

If the function of the kidney is considered to be primarily the regulation of the internal environment, then the introduction of water or hypertonic solutions into the body automatically subjects the organ to a task which it may or may not be able to fulfil. In the experiments in which water was administered, the kidneys of the adult animals proved themselves quite able to restore, if not absolutely to maintain, the constancy of this internal milieu. This was expected. The kidneys at birth, however, were not able to do so. When water alone was administered to newborn rats little was excreted and the tissue fluids must have remained correspondingly diluted. This was, however, evidently not a severe physiological handicap to the animals for they appeared little the worse, and were usually accepted by their mothers at the end of the experiment and reared successfully. By 4 days of age the animals were much better served by

their kidneys and excreted 20 % of the test dose in the first hour and altogether 35 % in 5 hr. Adult animals excreted from 40 to 60 % of the test dose in 5 hr.

To administer hypertonic solutions to animals which are already hydropenic, subjects them to a severe physiological strain. If the solution introduced is more hypertonic than the most concentrated urine which the kidney can produce, the kidney can only excrete the administered crystalloid by drawing upon some of the body water of which there is already a shortage. This is the situation which arises if a thirsty man drinks sea water. It is of benefit to the animal, however, that the kidney should draw upon the body water in this way, for in adult life the kidney can always excrete a much more concentrated solution than the animal could endure in its internal environment. In the urea experiments quoted in this paper the adults excreted about 27 % of the administered urea in the 5 hr. of observation; the newborn animals only 6 %. In the salt experiments the adults excreted 26 % of the dose; the newborn animals 5 %. Thus in both experiments the kidneys of the newborn animals showed themselves much less capable of dealing with these changed conditions. It is not a new observation that the kidneys of newborn animals are not such effective organs as they will become in adult life. It has been shown by Adolph (1943) that young dogs excrete water less rapidly than adult animals and the same is probably true of young children (Lasch, 1923), but these observations need confirmation. The kidneys of newborn rats, however, seem to be considerably less effective than those of newborn dogs or infants for they excrete no more than traces of the test dose. The sodium chloride and the urea clearances of human infants are known to be well below adult standards when the two are compared on the basis of surface area or body weight (McCance & Young, 1941; Gordon, Harrison & McNamara, 1942). These clearances have not been compared at similar periods of life in other animals but it is evident that such differences also exist in rats. Other peculiarities of renal function in the early days of life have been discussed by McCance (1946). These aspects of developmental physiology must be of some significance to anyone interested in the development and treatment of young children, but it may be some little time before they are fully explained.

SUMMARY

1. When 10 % sodium chloride solutions were administered by mouth to adult rats the animals responded by reducing the osmotic pressure of their urine and by producing a large diuresis which enabled them to excrete 27 % of the crystalloid in 5 hr. When a similar dose was given to newborn rats they responded by raising slightly the osmotic pressure of the urine, but they did not produce a diuresis and they excreted only 6 % of the test dose in 5 hr.

2. Hypertonic solutions of urea produced effects similar to those of NaCl in both the adult and the newborn rats.

3. When 5% of the body weight of water was administered to adult rats the diuresis was rapid and effective but newborn rats did not respond to similar treatment in the same way. They produced no significant diuresis and no dilution of the urine.

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THE ACTION OF ACETYLCHOLINE IN THE SUPRAOPTIC NUCLEUS OF THE CHLORALOSED DOG

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In an earlier paper (Pickford, 1939) experiments were described in which it was shown that acetylcholine (ACh.) given intravenously to the normal unanaesthetized dog during the course of a water diuresis resulted in a temporary inhibition of the diuresis. It was also demonstrated that this inhibitory effect could not be produced after the removal of the posterior lobe of the pituitary gland, and that the inhibition was unlike that caused by intravenously administered adrenaline, or by the lowering of the blood pressure due to inhalation of amyl nitrite. It was concluded, therefore, that this inhibition was caused by the action of ACh. on the central nervous system, inducing a liberation into the circulation of the antidiuretic hormone of the posterior pituitary body. If ACh. takes part in transmitting the impulse in the hypothalamus, the cells of the supraoptic nucleus at once come to mind as a likely place for the ACh. to be effective (Fisher, Ingram & Ranson, 1938). In order to test this suggestion the following experiments were performed.

METHODS

The method used was the direct injection amongst the supraoptic cells of small volumes of ACh. solution. Chloralose, given in the least possible dose, was employed as anaesthetic, as it has been shown (Theobald, 1934) that this substance interferes but little with the excretion of water.

Dogs and bitches were used. At a preliminary aseptic operation the kidneys were denervated by a thorough stripping of their pedicles, and the urethra made easily accessible by the dorsal slitting of the perineum in the female, and by exposing, slitting and stitching open the membranous urethra in the male. One to two weeks after operation the pituitary gland and optic chiasma were exposed through the mouth under soluble pentobarbitone anaesthesia. The soft palate was left unsutured. Early the next morning when the animal was well and lively, it was allowed a half pint of milk. Two hours later a hydrating dose of 250–300 c.c. water was given by mouth. Two to three hours later again 250–350 c.c. water containing 0·14 g. chloralose per kg. body weight were given by mouth, and urine collection begun by means of a soft rubber catheter passed into the bladder. When renal clearances were to be measured, ½–¾ hr. before the dose of water and chloralose 10 g. inulin in 50 c.c. 0·9% NaCl and 6 c.c. diodone were injected subcutaneously in the flanks. This subcutaneous administration was found to maintain, for some hours, adequate and fairly steady plasma concentrations of inulin and diodone. If creatinine was used, this was given by mouth in a 4 g. dose in 100 c.c. water at the time of the diodone administration.

After the water-chloralose mixture had been given, the dog became drowsy in 10–15 min., and in 30–40 min. no movement was elicited by any gentle manipulation. When drowsy, the dog was laid on its side on a sloping board, and its jaws held open by two adjustable metal rods attached to the board. With the help of a head lamp it was then possible, on retracting the soft palate, to obtain a clear view of the previously exposed pituitary and optic chiasma. When blood-pressure records were taken a cannula was inserted into one of the femoral arteries, with a saturated solution of sodium sulphate as anticoagulent. The injections into the supraoptic nuclei were made with a long fine needle bent at an obtuse angle $\frac{1}{2}$ cm. from the tip, and provided with a very short bevel. An insulin syringe was used, to the plunger of which was attached a screwhead such that a half-turn delivered 0·004 c.c. from the end of the needle. Solutions to be injected were freshly made each time in 0·9% NaCl to which a small quantity of India ink had been added. The volume injected was always 0·004 c.c.

At the end of the experiment the animal was rapidly killed by severing the great vessels with a scalpel through the thoracic wall. As a routine measure histological examination of the kidneys was always made. The base of the skull, with hypothalamus and pituitary in situ, was hardened in 5% formol saline (2% formaldehyde in 0·9% NaCl) for 24 hr., then the hypothalamus and pituitary dissected out, further fixed and hardened and blocked in paraffin. Serial sections 10 μ. thick were cut, every fifth section being kept and stained with toluidine blue. On examination the India ink granules showed the exact position of the injections.

Inulin was estimated colorimetrically after boiling with HCl and resorcin (Cole, 1943), diodone by the method of Alpert (1941), and creatinine by the alkaline picrate method.

<center>RESULTS</center>

In Fig. 1 (broken line) is shown the effect of injecting 8 μg. ACh. amongst the caudal cells of the left supraoptic nucleus of one dog, and in Fig. 2, at A, the effect on another dog of 40 μg. ACh. injected in a similar position. On 16 different occasions amounts of ACh. varying from 2 to 40 μg. were used. Two factors make accurate comparison of the effects of different doses of ACh. impossible. First, some of the ACh. solution was always lost to the exterior along the needle track, and second, there were considerable variations in the water load (Pickford, 1936) due to uncontrollable factors such as the course of the diuresis and the time available for observation before the anaesthetic became ineffective. Doses of 10 μg. ACh. given by this method produced a reduction in the rate of urine flow which varied from 80 to 91 %. This was the maximum reduction observed with any dose, though higher concentrations prolonged the effect. In this connexion it has to be remembered that, by this method, only some of the cells of one of the nuclei are subjected to stimulation.

On examination of the sections of the hypothalamus it was found that the injections had generally been made into the caudal part of the supraoptic nucleus, this being the more easily accessible. Sometimes, however, they were in the rostral part of the nucleus. In both instances ACh. led to a similar inhibition of the rate of urine flow.

Controls. In Figs. 3 and 1 (at B) may be seen the effect of control injections of 0·004 c.c. 0·9% NaCl amongst the cells of the supraoptic nucleus. There is either a transitory effect as in Fig. 3, or none at all as in Fig. 1, where the rate of urine flow continued its steady recovery from the ACh. inhibition and only

began to fall gradually 14 min. later, at a time when the normal disappearance of the water diuresis might be expected. It is unlikely that this delayed and slow falling off of the rate of urine flow was related to the hypothalamic injection as it in no way resembled the rapid and clear-cut responses seen when ACh. was used.
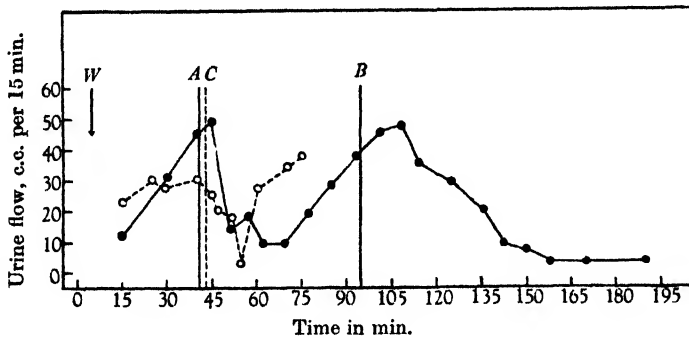
Fig. 1. Effect on the rate of urine flow of injections into the supraoptic nuclei of two dogs. ●—● At A, 8 μg. ACh. + 1 μg. eserine salicylate into the left supraoptic nucleus; at B, 0·004 c.c. 0·9 % NaCl into the right nucleus; o—o At C, 8 μg. into the left supraoptic nucleus. At W, 350 c.c. water containing chloralose was given.
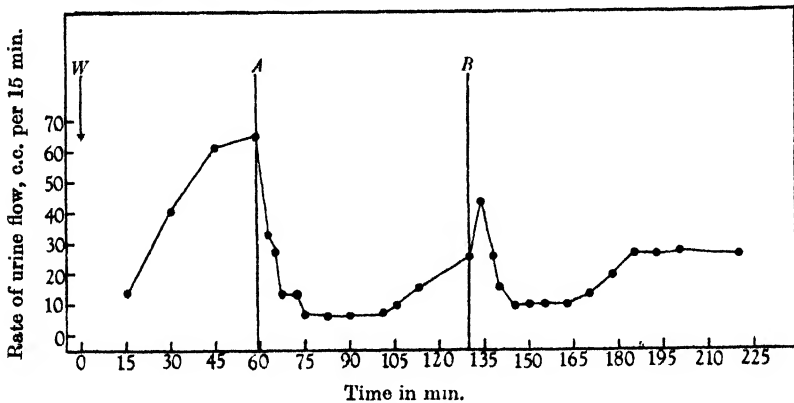
Fig. 2. Effect on the rate of urine flow of injecting, at A, 40 μg. ACh. into the left supraoptic nucleus, and, at B, 8 μg. eserine salicylate into the right nucleus of the same dog.

On one occasion 8 μg. ACh. + 4 μg. eserine salicylate were injected into the right mammillary body with no resulting change in the rate of urine flow. A similar injection made into the lateral hypothalamus was likewise without effect on the rate of urine flow.

Effect of eserine. If ACh. does take part in transmitting the impulse in the supraoptic nucleus, then the addition of eserine to the solution injected should prolong the action, or be effective by itself. In Fig. 1 (at A, continuous line), the result of injecting 8 μg. ACh. + 1 μg. eserine salicylate may be compared

with that following $8\,\mu$g. ACh. alone in another dog (broken line). In the former the time from injection to the beginning of recovery was about 30 min. and in the latter 12–15 min. In Fig. 4 the effect of ACh. with and without the addition of eserine may be compared on the same dog. At A, $7\,\mu$g. ACh. was injected into the left supraoptic nucleus and the rate of urine flow began to
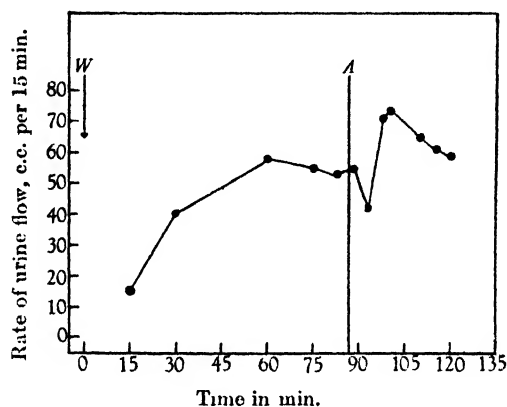
Fig. 3. Effect on the rate of urine flow of injecting, at A, 0·004 c.c. 0·9 % NaCl solution into the left supraoptic nucleus. At W, 350 c.c. water containing chloralose was given.
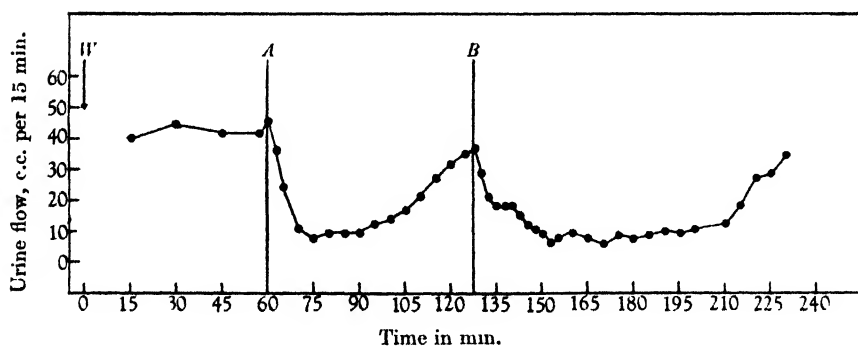
Fig. 4. Effect on the rate of urine flow of injection, at A, of $7\,\mu$g. ACh. into the left supraoptic nucleus; at B, of $7\,\mu$g. ACh. $+2\,\mu$g. eserine salicylate into the right supraoptic nucleus of the same dog. At W, 300 c.c. water containing chloralose was given.

recover from the resulting inhibition in 35 min. At B, $7\,\mu$g. ACh. $+2\,\mu$g. eserine salicylate were injected into the right supraoptic nucleus and the rate of urine flow did not begin to recover for 70–75 min. In this instance the inhibition following $7\,\mu$g. ACh. alone lasted longer than that after a similar dose in any other dog observed, where the time to recovery varied from 12 to 20 min. In this experiment, at the time of injection, very little of the solution was lost to the exterior and this may account for the more prolonged effect. In twelve observations the prolongation by eserine of the ACh. effect was constantly seen.

That eserine alone can bring about a reduction in the rate of urine flow is shown in Fig. 2 at *B*.

Blood pressure. In a number of instances blood pressure records were made during and after the injection of ACh. into the supraoptic nucleus. Sometimes small temporary variations in pressure were observed, but these showed no correlation with changes in the rate of urine flow.

Removal of the posterior lobe of the pituitary. If the inhibition of the urine flow on injecting ACh. into the supraoptic nucleus is due to a release of the antidiuretic hormone, then no inhibition should result from such an injection after the removal of the posterior lobe. Fig. 5 shows the result of an experiment in which, after preliminary procedures, the pituitary gland was removed. At *A* 8 μg. ACh. + 2 μg. eserine salicylate were injected amongst the caudal cells
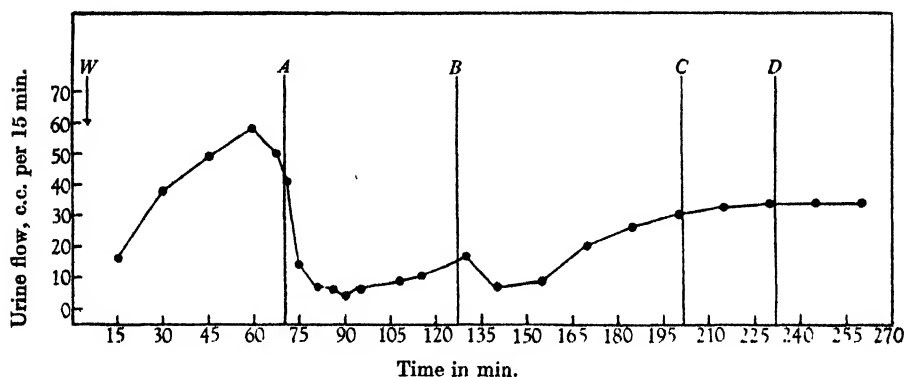
Fig. 5. Effect on the rate of urine flow of injection, at *A*, of 8 μg. ACh. + 2 μg. eserine salicylate into the left supraoptic nucleus; at *B*, removal of the pituitary gland; at *C*, similar injection into the right nucleus; and, at *D*, similar injection between the mammillary bodies. At *W*, 350 c.c. water containing chloralose was given.

of the left supraoptic nucleus and an inhibition of the rate of urine flow resulted. At *B*, during recovery from the inhibition, the whole pituitary gland and part of the stalk were removed. Polyuria had become established 1¼ hr. later. At *C*, a similar mixture of drugs was injected into the right supraoptic nucleus. The rate of urine flow was unchanged by this procedure. At *D*, the same mixture was injected between the mammillary bodies and had no effect on the rate of urine flow. Thus, in the period of polyuria immediately after the removal of the pituitary gland, ACh. applied to the supraoptic cells does not give rise to an inhibition of the rate of urine flow.

Renal clearances. On ten occasions, before and after the inhibition produced by the injection of ACh. into the hypothalamus, inulin or creatinine clearances were measured, and on six occasions the diodone clearances were simultaneously measured. The glomerular filtration rate was unchanged seven times; twice it fell slightly for a short time, and once it rose a little. The diodone

clearance during the period of inhibition of the rate of urine flow was maintained steadily at the pre-injection level on all six occasions.

It may be mentioned that in two dogs the inulin and diodone clearances have been measured, both when the animals were unanaesthetized, and after the administration of 0·14 g. chloralose per kg. body weight. In neither case did the degree of anaesthesia produced result in a change in the level of the clearances.

DISCUSSION

The experiments described above have shown that ACh. injected into the supraoptic nucleus of the chloralosed dog results in an inhibition of the rate of urine flow during water diuresis. This inhibition is prolonged by the addition of eserine salicylate to the solution injected and no systemic blood pressure changes can be associated with it. Eserine salicylate injected alone produces a similar inhibition of the rate of urine flow. The injection of 0·9 % NaCl into this same area has, at best, a fleeting effect. Further, eserine and ACh. injected together into one of the mammillary bodies or into the lateral hypothalamus are without effect on the rate of urine flow. No inhibition of the rate of urine flow was seen on injection of ACh. after the removal of the pituitary gland. All these observations were made on dogs whose kidneys had been denervated.

The foregoing results are not inconsistent with the hypothesis that ACh. stimulates the cells of the supraoptic nucleus, inducing thereby the release of the antidiuretic hormone from the posterior lobe of the pituitary. Further, these observations show that ACh. injected into the lateral hypothalamus or the mammillary bodies does not cause an inhibition of urine flow, which would suggest that these areas do not release an antidiuretic hormone, or that ACh. applied in this way does not release the hormone.

The method used is qualitative rather than quantitative. As has already been mentioned, some of the solution injected was always lost to the exterior along the needle track. Also, it is not known how many cells were affected by the diffusing ACh. The India ink particles can give no information on this point. It can only be said that higher concentrations of ACh. produced a longer-lasting inhibition of the rate of urine flow than did lower concentrations, and that if a dose were given above that necessary to produce an 80–91 % inhibition, the result was a further increase in the duration of the inhibition. On no occasion was a 100 % inhibition of the rate of urine flow seen. This is probably accounted for by the fact that the ACh. was applied to one nucleus only, and, in addition, reached only some of the cells of that nucleus. That the stimulation of some cells only of one supraoptic nucleus can produce so great an inhibition of the rate of urine flow is in agreement with the calculation of Hart & Verney (1934) that a fall of one part of antidiuretic hormone in $1·5 \times 10^{10}$ parts of plasma is probably a gross overestimate of the change in concentration necessary to bring about an appreciable alteration in the rate of urine flow in

man. There is agreement, also, with the observations that diabetes insipidus does not result if as few as 12–14% or 12–16% of the supraoptic cells are functioning (Magoun, Fisher & Ranson, 1939; Rasmussen & Gardner, 1940; Heinbecker & White, 1941; and Pickford & Ritchie, 1945).

Experiments are in progress to determine whether substances other than ACh. can stimulate the supraoptic cells, and whether ACh. is concerned in the activity of the posterior lobe itself.

SUMMARY

1. Experiments are described in which, during the course of a water diuresis in chloralosed dogs whose kidneys had previously been denervated, a solution of acetylcholine was injected directly into one or other supraoptic nucleus.

2. Acetylcholine injected into the supraoptic nucleus results in an inhibition of the rate of urine flow.

3. The inhibitory action of acetylcholine is prolonged by the addition of eserine salicylate to the solution injected. Eserine salicylate injected alone resulted in an inhibition of the rate of urine flow.

4. The inhibition of the rate of urine flow cannot be correlated with systemic blood pressure changes.

5. The inhibition was not seen after the removal of the pituitary gland.

6. No inhibition of urine flow followed the injection of acetylcholine into the mammillary bodies or lateral hypothalamus.

7. The results described are not inconsistent with the hypothesis that acetylcholine stimulates the supraoptic cells.

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REFLEX VASODILATATION IN HUMAN SKELETAL MUSCLE IN RESPONSE TO HEATING THE BODY

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Grant & Pearson (1938) studied the effect of warming the body on the blood flow in the human forearm. They found a small vasodilatation which they thought was in the skin. Wilkins & Eichna (1941) found a larger dilatation, and, since it appeared before any rise in the temperature of the skin of the opposite limb segment, they considered that it was deep to the skin, presumably in the muscle.

The object of this paper is to give further evidence for reflex vasodilatation in skeletal muscle in response to heat, in so far as it can be inferred from experiments on the blood flow in the forearm. Observations have been made on normal forearms, on sympathectomized forearms, on forearms with the skin flushed by mustard and on those with the skin blanched by adrenaline.

METHODS

Healthy men aged 20–35 years acted as subjects. Room temperature was about 20° C.

The subject stripped to the waist, bared his feet and lay with his back raised to an angle of about 45°. He was covered with a blanket. Both forearms were shaved. Plethysmographs for measuring the blood flow by Lewis & Grant's method were fitted (Barcroft & Edholm, 1943, 1945). Water-bath temperature was 35° C.

Blood flow was recorded at 5 min. intervals for about 30 min. Both feet were then immersed, up to the thickest part of the calves, in water at 44–45° C. The subject was covered with another blanket. Recordings continued at 5 min. intervals. The time of the beginning of sweating was noted. The experiment finished after the feet had been heated for 50 min.

The sympathectomized forearm. In this group of six subjects with unilateral upper limb sympathectomies the age limits, 13–61, were wider than those in the normal group, and three of the subjects were females. It is unlikely that this could have affected the significance of the results. The subjects' blood pressures were normal. Landis & Gibbon's (1933) body heating and finger temperature test was used to ascertain that the sympathectomy had been complete.

The flushed forearm. The forearm on one side only was used. After recording the blood flow for about 30 min., 15–20 g. of mustard (Colman's), previously made into a paste, was washed into the plethysmograph. Recording continued for about 30 min. To prevent the mustard from settling down to the bottom the contents of the plethysmograph were swirled round every 5 min. with an enema syringe.

The blanched forearm. Adrenaline was introduced into the skin by electrophoresis (Abramson, 1940; Barcroft, Bonnar, Edholm & Effron, 1943). A preliminary control electrophoresis experiment was done on each subject as follows.

The forearm on one side only was used. The plethysmograph was filled with adrenaline solution. (In recent experiments 0·5 g. adrenaline base was dissolved in 10 c.c. of H_3PO_4 of strength suitable to bring the pH to about 4·5. This was made up to 1500 c.c. with distilled water and warmed to 35° C. just before use.) The plethysmograph was not put into the water-bath, but the contents were kept at 35° C. by warming the outside every few minutes with a small gas flame, and swirling round the adrenaline solution by means of an enema syringe attached to a side-inlet. (The arrangements for electrophoresis were as shown in Fig. 1.)

Blood flows were recorded and determinations of the arterial blood pressure were made at 5 min. intervals for 30 min. Electrophoresis was then begun at 2–4 mA. and lasted for 80 min. Immediately after the experiment the plethysmograph was slipped off, and the skin of the forearm was examined. In recent experiments the circulation in the arm was then arrested for 3 min. and, after release, the treated area was watched to see if reactive hyperaemia developed.

Unsuccessful controls were of two kinds. In one, adrenaline was absorbed rather rapidly, causing increase in the blood pressure and in the forearm blood flow (Kunkel, Stead & Weiss, 1939). In the other, the skin was not properly blanched. In some subjects the fault was corrected by repeating the experiment with a different strength of the current.

In a successful control, very little adrenaline reached the general circulation. The blood pressure remained almost unchanged, only slight palpitation was felt, there was no tremor, and, above all, the forearm blood flow did not increase. Further, at the end of the experiment the skin of the part of the forearm which had been in the plethysmograph was either uniformly pale or else mostly pale with some cyanotic patches, and it was impossible to elicit reactive hyperaemia in it. (The reactive hyperaemia test was used in the last two experiments only.)

After a successful control the experiment was repeated later, with the difference that the feet were put into hot water for the last 40 min.
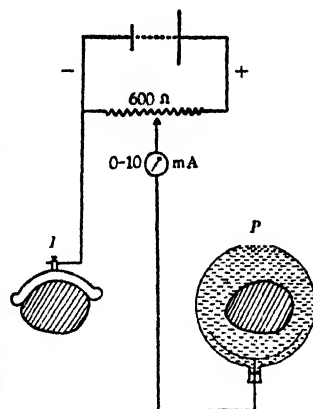
Fig. 1. Arrangements for the electrophoresis of adrenaline into the skin of the forearm. *P* is the plethysmograph containing the adrenaline solution and forearm. *I* is the indifferent electrode on one of the other limbs.

<div align="center">RESULTS</div>

Blood flow in the normal forearm. Typical effects obtained in six subjects are shown in Fig. 2. In these and all subsequent diagrams there is a preliminary period of 25 min. during which the flows are plotted at 5 min. intervals. After the 25th min. the time scale has been halved and the flows are plotted at 10 min. intervals. In all six subjects heating caused increase in the forearm blood flow. The average flows at the beginning and end of the heating were 3·1 and 9·3 c.c./100 c.c. forearm/min. respectively—a threefold increase. Fig. 3 shows a specimen record.

It is noteworthy that warming the body as above caused no significant discomfort. Sweating occurred in all subjects, beginning in 15–35 min. The final pulse rate was between 68 and 88 beats/min. The final mouth temperature was about 100° F.

Blood flow in the sympathectomized forearm. The results on six subjects with unilateral sympathectomy are shown in Fig. 4. Heating the feet had no effect on the blood flow on the sympathectomized side. The increase in flow in the normal forearms must have been mediated by sympathetic fibres from the vasomotor centre.
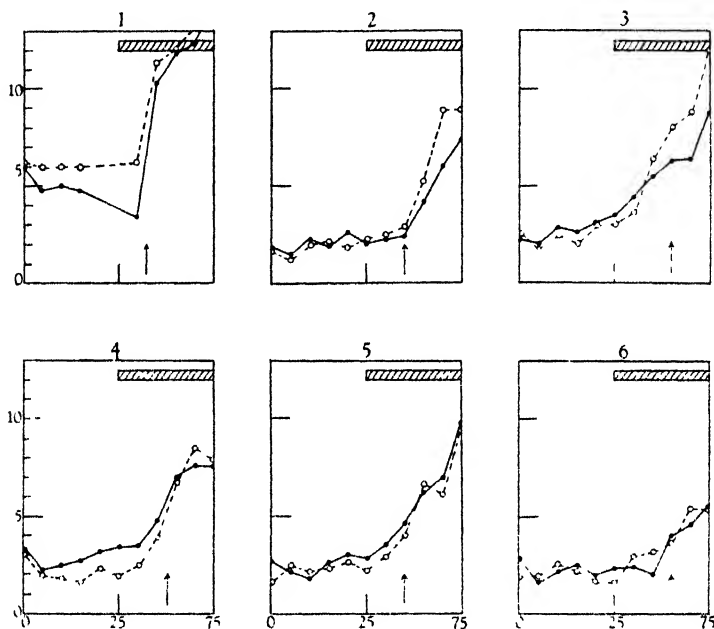
Fig. 2. Shaded rectangle: both feet in hot water. Dots and continuous line: right forearm. Circles and broken line: left forearm. Arrow: sweating. Ordinates: blood flow in c.c./100 c.c. forearm/min. Abscissae: time in min.
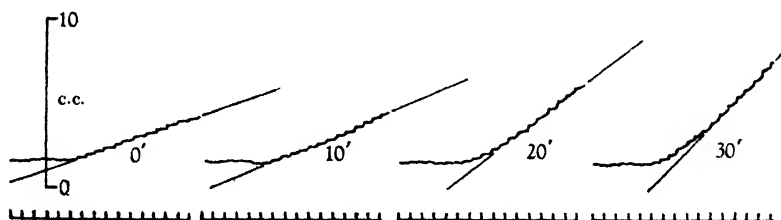
Fig. 3. Tracing recorded in the experiment shown in Fig. 2, No. 3. Left forearm 0, 10, 20 and 30 min. after immersion of both feet in hot water.

Blood flow in the flushed forearm. The mustard flushed the forearm skin, to a variable extent in all six subjects (cf. Killian & Oclassen, 1938). Fig. 5 shows that the blood flow in the forearm was increased in every subject. The average flow at the end of the experiments was 8·9 c.c./100 c.c. forearm/min.

The significance of this observation is as follows. The increase in blood flow caused by mustard (to 8·9 c.c.) was about the same as that caused by heating the feet (to 9·3 c.c.). In the mustard experiment the hyperaemia must have been mainly in the flushed skin. In the feet heating experiments, however, the skin was not flushed (confirming Grant & Pearson, 1938), a finding which suggests that the hyperaemia was in the underlying tissues.
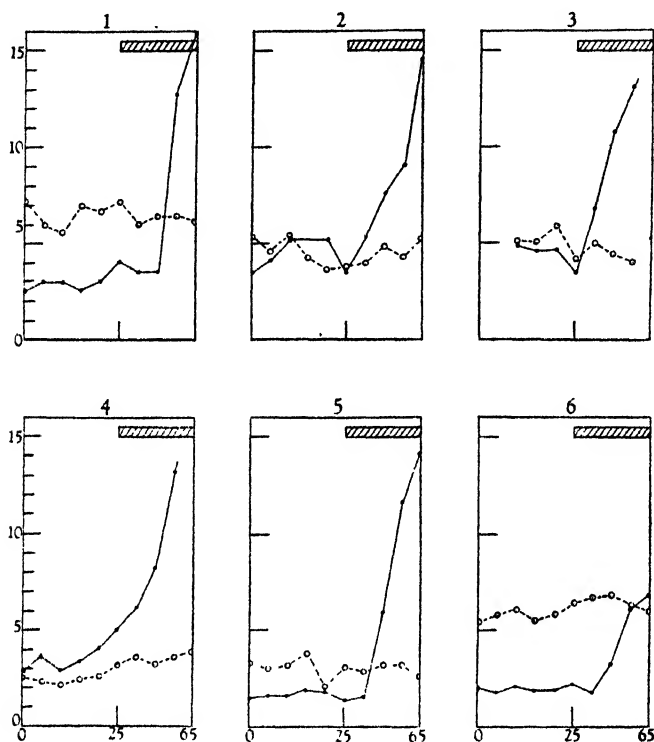
Fig. 4. Shaded rectangle: both feet in hot water. Dots and continuous line: normal forearm. Circles and broken line: sympathectomized forearm. Ordinates: blood flow in c.c./100 c.c. forearm/min. Abscissae: time in min.

Blood flow in the blanched forearm. The results obtained on six subjects are shown in Fig. 6. In five of them, heating the feet had the same effect as it had in the normal forearm, namely vasodilatation. (In one subject, Exp. 6, this was slight—as is occasionally found in a normal subject.)

Since adrenaline dilates the blood vessels of muscle (Kunkel *et al.* 1939), it was necessary to show that the vasodilatation was not due to the entrance of adrenaline into the general circulation or its passage through the subcutaneous tissue into the muscles. This was done in the control experiments, performed on the same subject with the same strength of adrenaline solution using similar
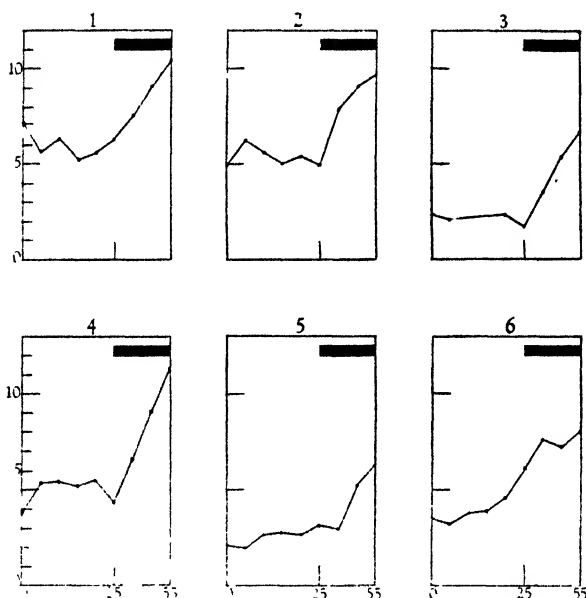
Fig. 5 Solid rectangle: mustard put in plethysmograph. Ordinates: blood flow in the forearm in c.c./100 c.c. forearm/min. Abscissae: time in min.
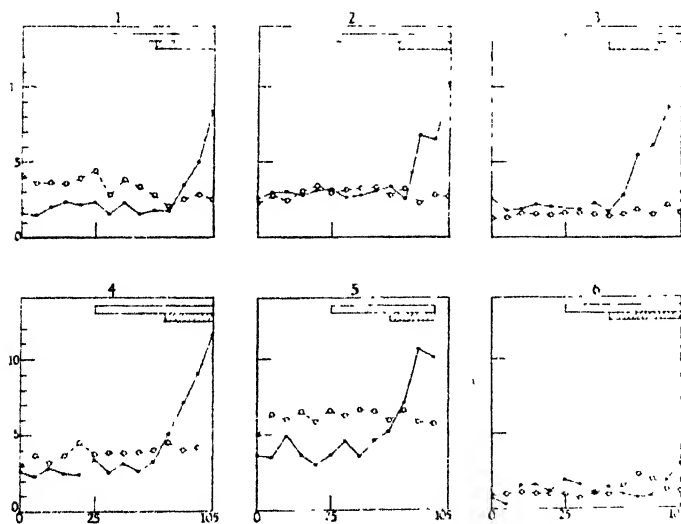
Fig. 6. Plain rectangle: adrenaline electrophoresis. Shaded rectangle: both feet in hot water. Dots and continuous line: forearm blood flow. Circles and broken line: forearm blood flow in control experiment with electrophoresis only. Ordinates: blood flow in c.c./100 c.c forearm/min. Abscissae: time in min.

current for similar time, without heating the feet. Fig. 6 shows that the electrophoresis had no effect on the forearm blood flow. The vasodilatation must therefore have been produced by body warming.

The accuracy of the conclusions to be drawn from these experiments depends on satisfactory occlusion of the cutaneous circulation. The appearance of the skin made it certain that the circulation in it was nearly negligible in every experiment in Fig. 6.

These experiments confirm Barcroft & Edholm's (1946b) suggestion that the vasodilatation produced by heat occurs in tissues deep to the forearm skin and most probably in the skeletal muscle which forms the bulk of the forearm.

DISCUSSION

Rein (1931) experimented on the effect of temperature on the blood flow through dog's muscles. Rise in air temperature from 18 to 45° C. induced vasoconstriction in the leg muscles. In view of the opposite finding in man, and of the disturbances known to be set up in the thermostromuhr by alteration of environmental temperature (Barcroft & Loughridge, 1938), the results of the animal experiments require confirmation.

Grant & Pearson (1938) recorded the forearm blood flow and heated the feet as described above. They found only a very slight change; the initial flow was, on the average 1·3 c.c. which increased on heating to 1·7 c.c. The corresponding figures in the experiments just described were 3·1 and 9·3 c.c. respectively. The difference between Grant & Pearson's results and our own lies in the use of different water-bath temperatures. In our experiments it was 35° C., in Grant & Pearson's it was 30° C. A bath at 30° C. cools the muscle and reduces the muscle blood flow and reactivity of the muscle blood vessels to such an extent that reflex vasodilatation is almost absent (Barcroft & Edholm, 1946a, Fig. 5).

Later Grant & Holling (1938) found that heating did cause reflex dilatation in the forearm and proved that it was actively excited in the vessels of the skin. This is, however, a thing apart, as in order to elicit it, much stronger body heating is required than that used in our experiments.

Wilkins & Eichna (1941) used a water-bath temperature of 32° C. and found that body warming brought about a two- to four-fold increase in forearm blood flow. This we can substantially confirm. They found also that the vasodilatation preceded the rise in temperature of the skin of the opposite forearm and they therefore thought that it must have been deep to the skin, presumably in the muscle. That the vasodilatation is for the most part in the muscle is fully confirmed by our results.

The question arises as to how the sympathetic brings about the dilatation. Is it a simple release of vasoconstrictor tone, or are vasodilator fibres involved? (Barcroft et al. 1943; Barcroft & Edholm, 1945). Release of constrictor tone

would increase the flow to approximately 8·3 c.c. (Barcroft *et al.* 1943). Body warming increases it to about the same extent, i.e. to 9·3 c.c. (see above). Heat therefore may act mainly by release of constrictor tone, but on general grounds, reciprocal decrease of constrictor and increase of dilator tone seems more likely.

It has been estimated that release of constrictor tone would increase the total muscle flow by about 1·5 l. (Barcroft *et al.* 1943). The effect of body warming would be of the same order. The vasodilatation in severe exercise is of course much greater, probably nearer 20 l.

It is curious that the hyperaemia through the hands and feet, when they are reflexly flushed by heat, is probably less than 1·5 l., i.e. the increase in flow through the skeletal muscles is probably greater than that through the extremities. (Each hand: vol. approx. 500 c.c.; increase in flow approx. 25 c.c./100 c.c./min.; increase per hand 125 c.c./min. Each foot: vol. approx. 1000 c.c.; increase in flow approx. 20 c.c./100 c.c./min.; increase per foot 200 c.c./min. Increase for both hands and both feet approx. 650 c.c./min.)

Per unit volume of tissue, however, the increase in the blood flow caused by body warming is far less in muscle than it is in the skin of the finger. Calculated per 100 c.c. of tissue it is of the order of 5–10 c.c. in muscle and 50–75 c.c. in finger skin. These facts help to explain why body heating causes a conspicuous rise in the temperature of the skin of the fingers (Landis & Gibbon, 1933) and yet has scarcely any effect on the temperature of the muscles. (In four experiments in which the forearm was exposed and had cooled for 1·5 hr., body heating raised the deep muscle temperature, on the average, from 32·0 to 33·2° C.; Bonnar, 1941.)

Reflex heating is used to increase blood flow in the hands and feet in various pathological conditions (Learmonth, 1943). Further work is needed to assess its value for inducing hyperaemia in the muscular system.

SUMMARY

1. The blood flow in the forearm (Lewis & Grant's plethysmographic method) was studied during body warming produced by covering the subjects with two blankets and putting both feet in water at 44–45° C. for 50 min.

2. Body warming increased the blood flow in the forearm on the average, from 3·1 to 9·3 c.c./100 c.c. forearm/min.

3. The effect was absent in sympathectomized forearms; it was a vascular reflex mediated by the sympathetic (confirming Wilkins & Eichna, 1941).

4. A flushing of the forearm skin, produced by mustard, increased the forearm blood flow to about the same extent as body warming. Since, however, body warming did not flush the skin it was likely that the heat vasodilatation was for the most part deep to the skin.

5. Body warming increased the blood flow in the forearm when the skin blood vessels were intensely constricted by adrenaline. It was concluded that

the dilatation was for the most part deep to the skin, most probably in the skeletal muscle.

6. The increase in total muscle blood flow during body warming, as above, would probably have averaged about 1·5 l./min.

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THE SURVIVAL OF TRANSPLANTED EMBRYO BONE GRAFTED TO CHORIOALLANTOIC MEMBRANE, AND SUBSEQUENT OSTEOGENESIS

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The extent to which bone transplants survive and the source of any new bone subsequently formed are still matters of controversy. Three main hypotheses have been put forward.

Barth (1893, 1895) came to the conclusion that all cells, layers, and tissues of transplanted bone perish, and that subsequent osteogenesis was brought about through the agency of host tissue. Since that time, the 'death and resurrection' theory has received the support of innumerable investigators. Leriche & Policard (1928) state: 'The question of the death of the transplant should be considered solved. It is 32 years since Barth conclusively shewed it. It is truly a waste of time still to seek to verify facts so well demonstrated.' Watson-Jones (1943) states: 'It is undoubted that the graft never participates in the circulation of the host.' Proponents of the 'death and resurrection' theory believe that boiled bone, on grafting, is entirely replaced, but the process is complicated by the relative impermeability of the graft to invasion by host tissue.

Ollier (1867, 1891) thought that periosteum, bone and marrow can under certain circumstances not only survive transplantation but that the newly formed bone which arises is formed by graft and not by host cells. Hey Groves (1939) inclines to this view. Macewen (1912), Matti (1936) and Mowlem (1944) employed small chip grafts, and interpret their excellent clinical results as suggestive of survival of the transplanted fragments.

In 1918 Nageotte demonstrated ossification in the region of some transplants fixed in alcohol. Polletini (1923) also observed occasional osteogenesis following transplantation of alcohol-fixed bone; he concluded that a substance with osteogenic properties diffused from the fragment. Levander (1938) transplanted small fragments of bone subcutaneously and examined the host area histologically from the second day onwards. He was of the opinion that

all grafted tissue perished, and that the new bone which formed in close association with the graft was produced as a result of the permeation of the surrounding host vascular connective tissue by a specific osteogenic substance originating in the graft. He then extracted callus with alcohol; on injection of the extract, osteogenesis sometimes occurred. In 1940 he repeated the experiments along similar lines, employing bone-marrow, freed of bone spicules as far as possible, with similar results. Annersten (1940, 1943) continued the experiments and improved the extraction technique; but several positive results occurred among his control experiments. He suggested the existence of an osteogenic hormone. Bertelsen (1944) repeated the work, and compared the potency of extracts obtained from corticalis, total bone, marrow, periosteum and epiphysis. In his experiments, marrow extract proved most potent. He also had positive results among his control experiments (liver). Attempts at concentration and purification were unsuccessful. Levander (1945) later extended the specific osteogenic hormone theory to cover many similar cases of tissue induction. Lacroix (1945) also has produced an alcoholic extract of cartilage capable of inducing osteogenesis on injection.

So far as experimental evidence of the fate of grafts is concerned, our knowledge depends upon subjective interpretation of histological and radiological appearances. The degree of radiopacity of a transplanted fragment is held to be a measure of its vascularization. Histologically, the criteria appear to have been the presence in sections of recognizable blood vessels containing blood cells; or the gross appearance of the bone fragment (a somewhat difficult tissue to preserve histologically) has been studied. The disadvantage in all previous experiments is that the grafts have been available for microscopical examination only in the fixed and dead condition.

The present account deals with the results of experiments in which the transplanted fragments could be examined microscopically in the living condition at any time.

In the past, a series of observations has been made on bone anlagen transplanted to the chorioallantoic membrane (Murray, 1926; Murray & Selby, 1932; Studitsky, 1936). These experiments, however, were directed towards a different end, and the technique employed did not allow of direct examination.

METHODS

Aseptic precautions were observed throughout. Fragments of os frontale of the embryo chick (14 days' incubation) were prepared. They measured approx. $2 \times 2 \times 0.5$ mm. and were placed either in buffered saline directly for use as live fragments, or were first transferred to boiling Ringer's solution for 30 sec. Samples of each batch of boiled bone were explanted as hanging-drop tissue cultures, and since no cell outgrowth occurred, it was assumed that no viable cells remained after this treatment.

A fragment of bone was placed on a sterile cover-slip in a small drop of cock plasma-chick embryo mixture; the ensuing clot served temporarily to hold the bone in position. The chorioallantoic membrane of another embryo (10 days' incubation) having been exposed by the customary

technique, the cover-slip bearing the fragment was inverted over the hole in the shell and sealed down with a beeswax mixture. The chorioallantois was then brought into apposition with the under surface of the cover-slip by inflation (Hoffstadt & Omundsen, 1940). The egg was then returned to the incubator in the vertical position in which it was maintained throughout.

For low-power microscopical examination the specimens were kept warm in a simple type of stage incubator and illuminated from one side by the focused image of a Pointolite lamp; for more critical work a Leitz Ultropak was employed with the same light source and incubator.

For histological purposes the bone and bone-bearing area of the membrane were excised and fixed in Carnoy's fluid after preliminary fixation with the same fluid in situ. Decalcification was carried out in Formol-nitric solution. Paraffin sections were generally stained according to Masson's Trichrome or Heidenhein's Azan technique. In some preparations filtered India ink was injected into the embryo circulation via a large membrane vessel. After fixation the bone-bearing area was excised, cleared in cedarwood oil, mounted and studied in toto.

Some technical difficulties were encountered. Critical examination showed that exposure of the membrane frequently led to capillary thrombosis in the exposed zone. Although this generally resolved within 24 hr., its presence was undesirable since survival of the bone fragments must depend upon re-establishment of a circulation within the briefest period. It was found that a few drops of 1/5000 heparin solution added to the shell membrane before exposure of the chorioallantois usually prevented capillary thrombosis.

In some eggs the membrane tended to sag away from the cover-slip, generally within the first 24 hr. of incubation. Under these circumstances, presumably according to the extent of clot liquefaction, the bone sometimes remained on the cover-slip (i.e. not in contact with the membrane) and inevitably dried up; sometimes it remained attached to the membrane and sagged away with it. The former preparations were discarded while the latter were 'pumped up' again as before until bone and membrane re-established contact with the cover-slip. The fragment generally remained in situ thereafter.

In spite of every precaution, some eggs became infected and were discarded.

RESULTS

The most striking observation concerned the remarkable rapidity with which a complete circulation became established within the live fragments. Of forty successfully transplanted live fragments, thirty-two were extensively vascularized within 18 hr. and the remainder within 48 hr. Of twenty-five boiled fragments studied, none were vascularized within 48 hr. A negligible circulation was present in one at 72 hr., but the remainder did not vascularize until a considerably longer period elapsed and, as will be described below, the appearance of the vascular patterns was quite different.

Observations on the living preparations

Live fragments. At the start of the experiment, primitive Haversian systems, often containing vessels full of stationary blood cells, could be seen in the bone fragment; the trabeculae of matrix and the bone-cell lacunae could also be seen. The richly vascularized membrane was naturally obscured in the region of the bone, where it was indented slightly, but elsewhere was clearly visible, and the circulation of blood cells within the vessels could easily be followed (Pl. 1, fig. 1).

After 5 hr. the indentation was less clear, and in many cases haemorrhage obscured the bone fragment. In one fragment, however, a slow but definite

movement of blood corpuscles along a vessel within a Haversian canal was already apparent.

At 18 hr. a marked change had occurred. Much of the extravasated blood had been removed and the entire fragments were penetrated by a complete system of vessels filled with circulating blood. Following the primitive Haversian canals, the vessels seemed to permeate the entire substance of the fragment. The vascular pattern of the surrounding membrane had also become augmented to some extent (Pl. 1, figs. 2, 3).

The fragment itself seemed to have sunk somewhat into the substance of the membrane so that the indentation had disappeared. Two main types of vessel were distinguishable. There were large tortuous sinusoidal channels through which the flow was relatively placid, and in which the lumen might carry perhaps 6–10 red corpuscles abreast, and there were also smaller and straighter capillaries, passing perhaps 1–3 corpuscles together at considerable speed. The size and course of any particular vessel seemed to be conditioned by the size of its canal; the vessels could easily be followed on their tortuous course through the matrix. The fact that leucocytes could be seen adhering to and rolling along the sticky endothelium showed that some local abnormality was present for the first few days.

Bone fragments were studied for as long as 10 days. Both resorption and deposition of matrix occurred. The precise details of the laying down and resorption of bone, particularly in relationship to the developing vascular pattern, are now being studied in more detail. In general terms it can be stated that growth in length or in width did not occur in the case of single grafts, but that the thickness of the fragment continued to increase. In many cases some peripheral resorption took place, which, together with central deposition, led to the formation of a sphere. The fragments remained fully viable until the host embryo hatched.

Boiled fragments. From the outset these appeared quite different. The matrix of the fragments seemed more refractile and granular and the Haversian contents were more opaque.

No vascularization occurred within 48 hr., although the vascular pattern of the membrane was somewhat augmented (Pl. 1, fig. 4). When vessels finally appeared within the bone, they were seen to consist of short and fine capillary leashes, which generally entered the bone vertically. The impression was gained that they simply penetrated the fragment by the shortest possible route. Little anastomosis within the bone was present until a late stage, and the final pattern was never so profuse as in the live fragments.

Large numbers of wandering phagocytes were present around and within these fragments.

Observations on fixed and stained material

Scrutiny of sections confirmed and amplified these findings. The unboiled fragments seemed perfectly normal. No cell degeneration was present and mitoses were seen among the osteoblasts. As in vivo, some of the cells seemed (on morphological grounds) to be actively osteogenic and others in a resting condition. In some places osteoclasts were seen. The vessels were in no way remarkable. The impression gained from sections of fragments after varying periods of incubation was, simply, that the fragment of bone had become incorporated in the host circulation and that modelling of the fragment was continuing (Pl. 1, figs. 5–7).

It was apparent that the fragments had shifted from their original situation, i.e. resting against the ectoderm, and had sunk within the substance of the membrane so as to become surrounded by mesoderm. Islands or 'pearls' of ectodermal cells were sometimes found within the marrow spaces of the fragments. In one case, such a pearl (Pl. 1, fig. 7) was traced in serial section to the ectoderm. Just beneath the fragments, but generally separated from them by one or more fusiform connective tissue cells, were syncytial masses, also of ectodermal origin. Danchakoff (1918) reported similar findings in spleen grafts. Goodpasture & Anderson (1937) evidently saw similar cells but described them as foreign body giant cells.

Sections of boiled fragments showed a different state of affairs. The matrix was shrunken and distorted with remains of cells and connective tissue present. Some cellular infiltration was apparent at 48 hr. (Pl. 1, fig. 8), and later a richly cellular and highly vascular connective tissue surrounded the grafts. There was little histological evidence of resorption and the boiled fragments seemed to be well tolerated. The reaction of the host tissue was rather slight, consisting chiefly of mononuclear cell infiltration; occasional osteoclasts were seen.

DISCUSSION

There are two possibilities regarding the manner in which the newly developed circulation arose from the membrane. First, it may have arisen completely de novo as capillary sprouts which pushed their way through the Haversian systems; secondly, newly formed and relatively short vascular buds may have grown up to, and fused with the cut ends of the vessels already present in the bone. The latter explanation seems the more likely; the time factor is in its favour, and the phenomena seem to parallel closely those observed in the junction of lymphatic vessels within transparent ear chambers (Clark & Clark, 1937). The early haemorrhages seen in the region of the graft thus probably arose as a result of the junction of a host capillary with one end of a graft vessel; a preliminary 'flushing' of the fragment would thus take place before

the establishment of a complete and closed vascular channel. This process was actually witnessed on one occasion, at the 5th hour.

Thus, under the present experimental conditions, which, essentially, predicate a highly reactive and vascular graft bed, it is safe to assume that transplanted fragments of live bone not only survived, but that, through the agency of their surviving cells they deposited fresh bone substance. The necessity for postulating a specific osteogenic inductor does not, therefore, arise in these experiments.

In the light of the foregoing it seems justifiable to suggest an alternative explanation for the occurrence of fresh bone in the grafting experiments of Levander (1938); namely, that some osteogenic cells within the fragment survived operation, and, having wandered out in their characteristic fashion from the fragment, brought about deposition of bone in the surrounding host tissue.

Fell (1932) demonstrated osteogenesis in tissue cultures 6 days after explanation of endosteal osteoblasts; Levander (1938) also observed it within 6 days of transplantation. In the present experiments trabecular thickening was often found after 4 days, a result which agrees well with the former observations. Osteogenesis following extract injection seems to develop only after a prolonged latent period and the results reported here shed no light upon the developmental history of such bone, nor upon bone formation following transplantation of dead tissue. The extent to which the results can be applied to the more general problem of osteogenesis is difficult to estimate. They constitute perhaps, evidence in support of the view that chip grafts survive and form osteogenic foci; they lay emphasis also on the need for delicate handling of transplant fragments.

SUMMARY

1. Grafts of living embryo bone, transplanted to chick chorioallantois, survive and rapidly become vascularized.

2. Boiled fragments do not become vascularized in a comparable manner.

3. Osteogenesis occurs in the former, but not in the latter.

4. It seems likely that short capillary buds developing from the host join up with vessels pre-existing in the transplanted fragment.

5. The application of these results to other work is discussed.
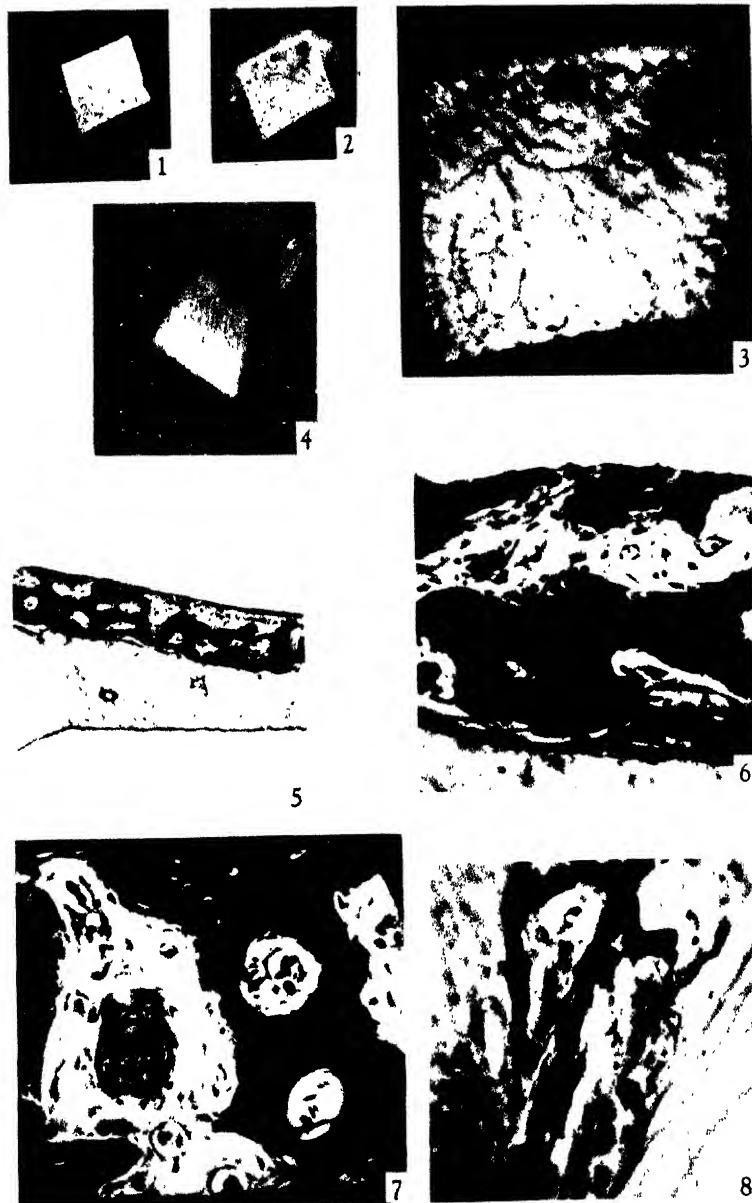
Figs. 1–8.

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EXPLANATION OF PLATE I

Fig. 1. Fragment of normal living bone at start of experiment. Outlines of primitive Haversian canals are visible. The chorioallantoic membrane can be seen around the fragment. × 6.

Fig. 2. Same fragment, 48 hr. incubation. The top right-hand corner is to some extent obscured by haemorrhage. Vessels can be distinguished within the fragment. × 6.

Fig. 3. Higher magnification of fig. 2. The course of vessels is easy to distinguish. Note ramification near lower left-hand corner. × 30.

Fig. 4. Fragment of boiled bone, 48 hr. incubation. Outlines of primitive Haversian canals can be seen but there is no vascularization. × 6.

Fig. 5. Vertical section of normal bone on membrane, 24 hr. incubation. Masson Trichrome stain. × 50.

Fig. 6. Similar, 3 days' incubation. The bone appears normal. × 400.

Fig. 7. Similar, 8 days' incubation. A primitive Haversian canal is seen. An 'ectodermal pearl' occupies a central position. Above and to its left, active osteoblasts are present: below, an osteoclast. Two small canals containing capillaries are present to the right. × 400.

Fig. 8. Boiled bone, 48 hr. incubation. Shrunken and distorted lamellae are visible. Commencing cellular infiltration of bone. × 400.

THE MECHANICAL AND STRUCTURAL PROPERTIES OF BONE IN RATS ON A RACHITOGENIC DIET

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In earlier investigations of bone strength (Bell, Cuthbertson & Orr, 1941; Bell & Cuthbertson, 1943) it was not found possible to produce changes in the quality of bone material either by reducing the calcium intake or by administering various hormones. Since in these experiments the percentage of ash in all the bones remained relatively constant it was decided to set up further experiments in which the ash content of the bones could be reduced by withholding vitamin D. Estimations of breaking stress as an index of bone quality were to be made at the end of the experiment, and, in addition, the crystalline structure of the bone material was to be studied by X-ray diffraction technique.

METHODS

Diet

In order to produce as marked rachitic changes as possible, the Steenbock diet (No. 2965) (Steenbock & Black, 1925), consisting of ground maize, wheat gluten, $CaCO_3$ and NaCl, was used because the constituents were available even in 1942. The animals, male albino rats, were obtained from Messrs Boots Ltd. After weaning at 3 weeks they were kept on a full diet (Boots B16) until they reached roughly 50 g. in weight, at an age of about 4 weeks. One of each litter was then killed (group C, Tables 1, 2), the stomach and intestines were removed, and the carcass ashed and analysed for Ca and P. One of the remainder was put on the rachitogenic diet with a supplement of vitamin D (group N); the remaining animals (one or two) were placed on the rachitogenic diet without vitamin D (group R). In all three experiments the rats of groups R and N were kept in individual cages and a record kept of the food supplied to each animal.

In the first experiment group N received 3 drops cod-liver oil (Seven Seas) daily; group R received 3 drops olive oil daily. In the second experiment the procedure was as in the first experiment but, in addition, all animals received 0·2 c.c. 'Ephynal' (Roche) subcutaneously 3 weeks after the beginning of the experiment and 0·5 c.c. a week later. This is a total dose of 35 mg. vitamin E.

In the third experiment the rats of group N received the Steenbock diet with vitamin A, calciferol and vitamin E; those of group R received the Steenbock diet with vitamin A and vitamin E only. The vitamins were all administered from the first day of the experiment; vitamin A acetate (obtained from British Drug Houses Ltd.; recrystallized to get rid of vitamin D so far as possible) was given orally in a dose of 40 i.u. in arachis oil twice a week; 10 i.u. calciferol (Glaxo Ltd.)

were given twice weekly orally; 20 mg. vitamin E (Roche) per week were administered sub-cutaneously. In addition, a further group S, consisting of one rat from each litter, was fed on the Rowett Institute stock diet (Thomson, 1936) ad lib. The rats of group S were kept together in a large cage and no record of food consumption was made.

Analysis of the Steenbock diet given to groups N and R in Exps. I–IV gave the following values: 1·235 g. Ca and 0·236 g.P/100 g. diet. The Ca/P ratio of the food was therefore 5·24 to 1.

Table 1 summarizes the experimental groups of rats.

TABLE 1. Experimental groups of rats

Group	Basic diet	Supplements				
		Exp. I	Exp. II	Exp. III	Exp. IV	Exp. V
C	Killed at the beginning of the experiment					
R	Rachitogenic	Olive oil	Olive oil Vitamin E	Vitamin A Vitamin E	Egg albumin Vitamin A Vitamin E Vitamin C to half of rats	Vitamin A
N	Rachitogenic	Cod-liver oil	Cod-liver oil Vitamin E	Vitamin A Vitamin D Vitamin E	Egg albumin Vitamin A Vitamin E Vitamin C to half of rats Vitamin D	Vitamin C to half of rats Vitamin A Vitamin D
S	Good stock diet			None		None

Mechanical tests

The tests for measuring breaking stress on bending and on twisting were carried out exactly as described in the first paper of this series (Bell *et al.* 1941). In all cases the right femora were used for the bending tests while the left were used for the twisting tests. The breaking stress on bending was calculated from formula (3) of the above paper, viz. $S = M - \left(\frac{\pi}{32} \frac{ab^3 - dc^3}{b} \right)$.

Chemical methods

The methods of preparation and of analysis of bones and carcasses already described, were again used (Bell *et al.* 1941). The Ca of carcasses and food was estimated by Shohl & Pedley's method (1922). The carcasses were first dried in an oven and then ashed in an electric muffle furnace. When the mechanical tests were completed some of the femoral fragments were retained for X-ray crystallography; the remainder of the bone fragments were ashed in a crucible at full Bunsen heat and then dissolved in N-HCl with heating on a boiling water-bath for 2 hr. The Ca content was estimated by the Clark-Collip modification of the standard micro-method for serum Ca (Peters & van Slyke, 1932). In Exps. I and II the phosphate estimation on carcasses was done by the uranium acetate titration method (Peters & van Slyke, 1932). The phosphate of all bone samples, and the phosphate of the carcasses in Exp. III, was estimated colorimetrically after precipitation with strychnine molybdate (Peters & van Slyke, 1932). The readings were made on a photoelectric colorimeter (Bell & Guthmann, 1943).

X-ray crystallographic methods

All the specimens were mounted on a non-rotating holder with the direction of the bone length perpendicular to the incident X-ray beam and exposed to Cu $K\alpha$ radiation with a specimen to film distance of 4·01 cm.

19—2

Intact specimens. Small portions of approximately 10 by 0·2 by 0·2 mm. were cut from the fragments of the central area of the femoral shaft. The location of the selected portion was standard throughout all the experiments.

Decalcified specimens. Several methods are available for effecting decalcification, but it is necessary to know first if the collagen of the bone remains unaltered in spite of the chemical treatment required. The diffraction pattern of fresh rat-tail tendon was compared with the pattern obtained after it had been immersed in the decalcifying fluid for the time necessary to decalcify the small bone specimens. Neither dilute ammonium citrate (White's solution) nor 1% formic acid caused any change in the orientation of the collagen pattern. Both methods were adopted in the routine decalcification of specimens. All such specimens were examined at room humidity.

Deproteinized specimens. The bone specimens were cut to dimensions suitable for X-ray examination, boiled in water for 24 hr. and then subjected to trypsin digestion at pH 8 for 4–5 days. After thorough washing it was shown by Kjeldahl's method that the specimens contained no N: digestion was therefore complete.

Assessment of orientation. The 0002 reflexion (Tables 4, 5) is the most intense oriented reflexion occurring in the intact bone diffraction pattern. The angular measurement of this diffraction arc was taken as a measure of the degree of orientation shown by the inorganic crystallites.

RESULTS

The nutritional, chemical and physical results are summarized in Tables 2 and 3.

Nutritional results

The recorded values of food intake in Table 2 are slightly in excess of the actual amounts ingested owing to spilling. The animals fed on the basic rachitogenic diet plus vitamin D (group N) ate more food and their retention of Ca and P was higher than that of the rachitic animals (group R). Group N showed a slow but continuous increase in body weight, while group R showed some loss of weight towards the end of all the experiments (Fig. 1). The group of animals (S of Exp. III) which were fed on a good diet looked more healthy, grew more rapidly and retained more Ca and P than the other groups.

Three weeks after the beginning of Exp. 1 all the group R rats showed increasing weakness of the hindlegs, progressing in some cases to almost complete paralysis in which the hindlegs were dragged along behind the body. The legs were not tender. This sign was not shown by group N. Since this paralysis could conceivably interfere with bone growth an attempt was made to eliminate it from Exp. II by giving vitamin E. This was not successful. At the end of this experiment two of the R rats showed slight weakness of the hindlimbs and two others had complete paralysis of these limbs. A further attempt to avoid paralysis was made in Exp. III by adding vitamin A as well as vitamin E to the basic diet. As before, group N showed no paresis; but in group R severe paralysis was seen at the end of the experiment in five rats and some weakness in another four; only two showed no paresis of the hindlimbs. It is our impression that the animals of group R in Exp. III were in poorer condition at the end of 30 days than the corresponding animals of Exp. II at the end of 36 days.

TABLE 2. Dietetic history of rats; R, on a rachitogenic diet; N, on a rachitogenic diet supplemented with vitamin D; S, on a natural complete diet. C are controls killed before the start of the diet

Group	No. of rats	Total food intake per animal (g.)	Total Ca intake (g.)	Total P intake (g.)	Body wt. (g.) Initial	Body wt. (g.) Max.	Body wt. (g.) Final	Ca content of gutted animals (g.)	P content of gutted animals (g.)	Retention per animal Ca (g.)	Retention per animal P (g.)	Percentage retention of intake Ca	Percentage retention of intake P
Exp. I; began 3. iv. 42; duration 27 days													
R	10	170	2·10	0·401	46·5	68·5	60·5	0·289	0·255	0·030	0·007	1·4	1·7
N	7	183	2·26	0·432	45·0	74·5	74·5	0·440	0·343	0·181	0·095	8·0	22·0
C	7	—	—	—	46·5	—	—	0·259	0·248	—	—	—	—
Exp. II; began 22. v. 42; duration 36 days													
R	8	217	2·68	0·512	50·0	71·0	64·0	0·341	0·274	0·034	0·004	1·3	0·8
N	8	271	3·35	0·640	49·5	85·5	84·5	0·553	0·412	0·246	0·142	7·4	22·2
C	6	—	—	—	49·5	—	—	0·307	0·270	—	—	—	—
Exp. III; began 9. x. 42; duration 30 days													
R	11	210	2·59	0·496	50·5	73·0	65·5	0·322	0·247	0·021	0·015	0·8	3·0
N	7	244	3·01	0·576	51·5	88·0	88·0	0·526	0·383	0·225	0·151	7·5	26·2
C	7	—	—	—	50·0	—	—	0·301	0·232	—	—	—	—
S	6	—	—	—	45·5	141·5	141·5	1·250	0·804	0·914	0·572	—	—

TABLE 3. Chemical and physical properties of bones from rats, R, on a rachitogenic diet; N, on rachitogenic diet supplemented with vitamin D; S, on complete diet

Group	Wt. of femur (g.)	Length of femur (cm.)	Ash in femur %	Ca in ash %	P in ash %	Ca in femur %	Ca/P ratio in femur	Greatest ext. diam. a 0·001 in.	Least ext. diam. b 0·001 in.	Thickness of wall t 0·001 in.	Bending moment at fracture M (in. lb.)	Twisting moment at fracture T (in. lb.)	M/abt	T/abt	Breaking stress lb./in.^{2} formula (3)
Exp. 1															
R	0·114	1·92	36·7	34·3	17·6	12·6	1·95	95·6	81·2	6·88	0·360	0·309	7·15	5·19	11,710
N	0·130	2·28	42·5	35·1	17·5	14·9	2·01	107·4	88·1	8·67	0·837	0·531	11·33	6·22	18,700
Exp. II															
R	0·126	2·06	34·5	38·6	18·4	13·3	2·10	99·3	81·7	7·59	0·429	0·419	8·04	4·95	12,850
N	0·158	2·46	42·6	36·4	18·4	15·5	1·98	109·7	92·0	8·98	0·981	0·772	10·79	9·15	18,130
Exp. III															
R	0·115	1·99	38·5	36·9	18·3	14·2	2·01	97·9	82·7	7·05	0·436	0·342	6·97	6·53	11,860
N	0·154	2·37	45·0	37·1	18·3	16·7	2·04	109·3	88·2	8·76	0·952	0·602	10·55	8·43	18,590
S	0·288	2·73	59·7	38·5	18·6	23·0	2·07	127·0	102·1	16·60	2·683	1·670	12·61	8·05	26,890

Methods used to determine a, b, t, M and T, and the breaking stress are described in full by Bell, et al. (1941).

Skeletal data

In all cases straight X-ray photographs of group R carcasses showed gross evidence of rickets, while the epiphysial lines in group N were quite narrow. The average weights of the femora in the R groups were from 12 to 26 % less than those of the femora in the N groups. In Exp. III the average femoral weight in group S was 90 % greater than in the corresponding N group. The
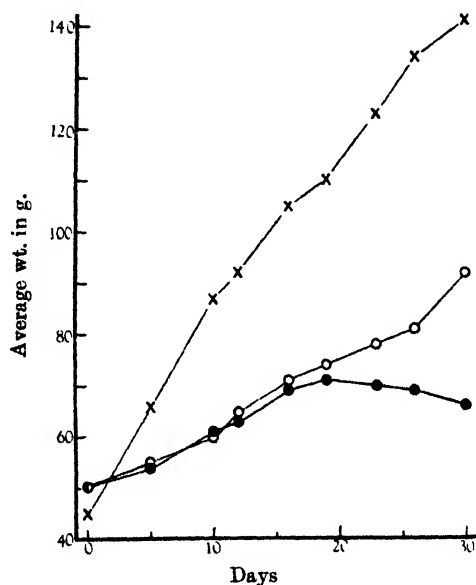
Fig 1. The average growth curves of the three groups, R ●, N ○, and S ×, of Exp. III.

average lengths of the R group femora in Exp. I, II and III were 16 % less than the corresponding N femora; S group femora were 16 % longer than the corresponding N group bones. The ash content of the R femora was on the average between 14 and 19 % less than that of the N femora; the S group had 33 % more ash than the N group. The percentages of Ca and P in the ash of all the bones were very nearly constant. For this reason the Ca/P ratios in the bones were nearly 2·0 in all cases.

The external diameters of the centre of the shaft (a, b) of the R femora were about 10 % less than those of the N femora; the S group were about 16 % greater externally. The thickness of the wall (t) of the femora at the midpoint of the shaft was about 18 % less than the wall in group N; the wall in group S femora was 90 % thicker than that of the corresponding N group. (For an exact definition of a, b and t see Fig. 2 of Bell, et al. 1941.)

The bending moment required to break the R femora was 54–57 % less than that for the N group. The bones of group S were 182 % stronger than those of

group N. In twisting the R femora were 42–46% weaker than the N femora; the S femora were 178% stronger.

In Fig. 2 all the observations on breaking stress on bending (calculated by formula (3)) have been plotted against the percentage of ash in the bone fragments. The correlation coefficient r is $+0.71$ (s.e. $=0.074$). It should be noted that both of these values are independent of the actual size of the individual bones; they may be regarded as indexes of the quality of the
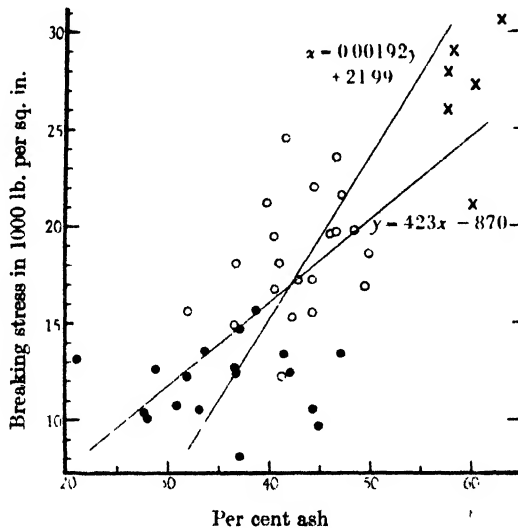
Fig. 2. Scatter diagram showing the relationship between the breaking stress in 1000 lb./sq.in. and the percentage ash content of femora from rachitic (R) animals ($\bullet$), from group N animals ($\bigcirc$), and from animals on an adequate diet ($\times$). The correlation coefficient $r=0.71$. The equations to the regression lines are given, where y is the breaking stress in lb./sq.in. and x is the percentage of ash.

material composing the bone. The correlation between the percentage of ash in bone and the breaking stress on bending, calculated from the M/abt formula, is $+0.52$ (s.e. $=0.15$). The difference between these two values of r is not significant. The correlation between bone-ash percentage and the breaking stress on twisting is $+0.405$ (s.e. $=0.12$).

The ratio of breaking stress on twisting to breaking stress on bending has been calculated for the forty pairs of femora in which both measurements were available. There is no significant difference (using Fisher's t test) between the average values of this ratio in each of the three groups R, N and S.

Results of X-ray crystallography

Specimens from the femora of all the rats in Exps. III and V were examined. For details of Exp. V see below. The spacings measured, indices assigned and the orientation shown by the diffraction rings are tabulated for rachitic (R)

specimens (Table 4) and for control (S) specimens (Table 5). The corresponding data for deproteinized specimens are given in Table 6. Typical X-ray diffraction patterns given by these specimens have already been published (Dawson, 1946), and the relation of the oriented diffraction arcs to the bone crystallite position discussed.

TABLE 4. X-ray crystallographic measurements from bone specimens from rats on rachitogenic diet (group R)

Ring no.	$d*$ (A.)	Indices	Orientation
1	12·8	Organic spacing	Equatorial
2	8·30	$01\bar{1}0$	Equatorial
3, 4	5·4 to 4·2	Organic, $01\bar{1}1$, $11\bar{2}0$	None
5	3·44	0002	Meridional
6, 7	3·17	$01\bar{1}2$, $12\bar{3}0$	None
8, 9	2·87	$12\bar{3}1$, $03\bar{3}0$	None
10	2·69	Organic spacing	Meridional

* All spacings correct to ± 0.05 A.

TABLE 5. X-ray crystallographic measurements from untreated specimens of normal bone from control (S) rats on complete diet

Ring no.	$d*$ (A.)	Indices	Orientation
2	8·30	$01\bar{1}0$	Equatorial
3, 4	5·3 to 4·2	Organic, $01\bar{1}1$, $11\bar{2}0$	None
5	3·44	0002	Meridional
6, 7	3·17	$01\bar{1}2$, $12\bar{3}0$	None
8, 9	2·87	$12\bar{3}1$, $03\bar{3}0$	None
10	Absent	—	—

* All spacings correct to ± 0.05 A.

A comparison of the data for untreated bone specimens given in Table 4 and Table 5 shows that: (1) The inorganic spacings have the same value, within the limits of experimental error, in control (S group) and in rachitic specimens (R group). (2) The organic spacings 1 and 10 occur in rachitic specimens only. The broad diffraction ring from 5·4–4·2 A. in the rachitic specimens is reduced to 5·3–4·2 A. in the control specimens. This ring has been shown to be composed of an organic spacing with the inorganic $01\bar{1}0$ and $11\bar{2}0$ superimposed. Its weaker intensity in the control specimens is due to a decrease in the scatter from the organic constituent; the inorganic spacings remain the same in intensity in the rachitic and control bones, whereas organic spacings 1 and 10 do not appear in the control.

From Table 6 (deproteinized specimens) it is seen that, on removal of protein, there is a considerable sharpening of the inorganic diffraction pattern, without, however, any appreciable change in the spacings measured. The composite diffraction rings 3 and 4, 6 and 7, and 8 and 9 in the untreated specimen are resolved in the deproteinized specimens. The decrease in intensity of the diffraction rings with decrease in spacing is much more marked in the case of untreated specimens. In deproteinized specimens there appear two further

unoriented diffraction rings 10 and 11, whereas, with untreated rachitic bone, only the oriented organic spacing 10 occurs (Tables 4, 6).

The organic spacings measured from the decalcified specimens (Table 7) are very similar to those given by Astbury (1940) for collagen and gelatin, for which structures have been proposed by Astbury & Bell (1940).

TABLE 6. X-ray crystallographic measurements from deproteinized bone samples

Ring No.	d (A.)	Indices	Orientation
2	8·14	01$\bar{1}$0	Equatorial
3	5·28	01$\bar{1}$1	None
4	4·22	11$\bar{2}$0	Equatorial
5	3·45	0002	Meridional
6	3·31	01$\bar{1}$2	None
7	3·22	12$\bar{3}$0	Equatorial
8	2·96	12$\bar{3}$1	None
9	2·86	03$\bar{3}$0	Equatorial
10	2·72	02$\bar{2}$2	None
11	2·55	03$\bar{3}$1	None

No differences were observed between the bones of rats on rachitogenic diet, or rachitogenic diet supplemented with vitamin D or on a complete diet.

TABLE 7. X-ray crystallographic measurements obtained from decalcified bone specimens

Spacing (A.)	Orientation
11·2–13·8	Equatorial
4·1– 5·5	Slight Equatorial
2·8– 2·9	Meridional

No differences were observed between the bones of rats on rachitogenic diet, or rachitogenic diet supplemented with vitamin D or on a complete diet.

In both rachitic and normal bone there is uniaxial orientation of both organic and inorganic material in a direction parallel to the bone length. This is most clearly indicated, in the case of the inorganic material, by the most intense oriented 0002 spacing (Tables 4, 5). The orientation is most pronounced at the centre of the shaft and decreases towards the epiphysial area where there is no detectable orientation. At the centre of the shaft there is no significant difference in the orientation shown by the rachitic and normal specimens.

DISCUSSION

Chemical and mechanical findings

The differences in weight of the femora in the three groups of rats are roughly in accordance with those of body weight; the femur lengths are similarly, but less closely, related. The food intakes, and the retentions of Ca and P, are consistent with the poor growth which occurred in all but the animals on the stock diet.

Analytical values for percentages of Ca and of P in ash, and for Ca/P ratio, show no notable differences between the groups. The values for the percentage of ash in the femur are of the order reported elsewhere for rats on Steenbock

and similar diets (Jones, 1939; Shohl, Fan & Farber, 1939; Shohl & Farber, 1941; Irving, 1944). Although the average values for the percentage of ash in the femora of R animals show differences from those of N animals, these are not large, and are much smaller than the differences between N and S rats. Furthermore, results for the percentage ash in the femora of individual animals of the R and N groups showed values for some N animals lower than those for some R animals. The degree of rickets produced in the R rats was always gross according to skiagrams of the epiphyses but was far from uniform if judged by ash content. With one minor exception, the N rats showed no definite skia-graphic evidence of rickets in spite of the great variation of the values for percentage ash in femora in the group. It was hoped, when the experiment was designed, to produce two groups of rats with clear cut differences in the chemical composition of the bones. The moderate success achieved was pre-sumably due partly to the individual variations in the response of the rats, and partly to the well recognized inadequacy of the Steenbock diet which is deficient in many important nutrients in addition to vitamin D (O'Brien & Morgareidge, 1938; Jones, 1939; Dodds & Cameron, 1943; Irving, 1944). The latter explanation is supported by the slow rate of growth of both R and N animals and by the occurrence of paralysis in many of the R animals.

In view of these difficulties it may be best to regard the Steenbock diet simply as a means of interfering with normal bone growth and bone composition rather than as a cause of uncomplicated rickets. For this reason all the results of the mechanical tests have been compared with the corresponding ash analyses in Fig. 2. The correlation found is fairly high when the difficulties of measuring breaking stress on small irregular objects are allowed for. The correlation between breaking stress on twisting and ash percentage is less. This is not unexpected, since the difficulties in measuring twisting stress are much greater; in our previous work the values for breaking stress on bending were always more consistent. Since the correlation between bone strength and bone ash is high there is no good reason to suspect that breaking stress (i.e. bone quality) is dependent on any factor other than ash content.

The ratio of breaking stress on twisting to breaking stress on bending was the same for R, N and S animals. There is thus no reason to suppose that the fundamental structural association between the organic and inorganic material differs in the three groups. The meaning of this ratio has already been discussed (Bell *et al.* 1941, p. 313).

Note on the absence of rachitic deformities

The bowing of the limbs seen in human and canine rickets is usually explained as being due to the weakness of the bones which give way under the weight of the body. While the weight of the body is undoubtedly the force bringing about these deformities the manner of their production cannot be dismissed with such a simple explanation for two reasons.

First, the femora of the rachitic rats (R) in the present experiment were about half as strong as those of protected rats (N), and only about one-fifth as strong as the femora of the well-fed group S as will be seen from the values of M in Table 3. The bone material of group R was only half as good, as regards quality, as that of group S (see last column of Table 3, breaking stress). There is thus no doubt that the rachitic bones are weak, but, in spite of this, not one showed any rachitic deformity. Indeed, rachitic deformities are never encountered in the rat. Our previous experiments furnish additional evidence that weakness by itself does not result in permanent bending; the very thin and fragile bones, but with normal breaking stress, of rats on an extremely low Ca intake were of normal shape (Bell *et al.* 1941).

In the second place the bones of our rachitic animals have, in spite of their weakness, a considerable factor of safety, since none showed any fracture under the stresses set up in them during the activities of feeding and of exploration of their cages, or even in accidental falls. Curves of deflexion against load, obtained from the bending test applied to rachitic bones, show that the bones are elastic up to a very severe load, i.e. the bones show very little ductility, or set, unless the stresses are nearly at the maximum which can be tolerated without producing a fracture.

The mechanical properties of rachitic bones of man and the dog have not yet been investigated, but it is of interest to see what conclusions emerge if we assume that the above considerations apply to them just as to the rat, i.e. if we assume that although the bones are weak in rickets they are never likely to be stressed beyond their elastic limit. The rachitic deformities occurring in man and the dog cannot on this assumption be explained on simple static structural properties such as those which would be considered by an engineer dealing with a steel tube; the deformities are more likely to be due to an abnormal reaction of the living animal to the bending forces, perhaps best described as a disorder of bone modelling. It is, however, not easy to imagine why one species should show rachitic deformities and another none at all.

The cause of the paralysis

The occurrence of paralysis of the hindlimbs in most of the R animals was an unexpected and unwanted complication. The same condition has been reported by Shapiro (1941) after 6–9 weeks on the Steenbock diet. This is later than the onset in our experiments. Neuromuscular disturbances of various sorts have been recorded by others who have fed rats on low Ca or low P diets (Schneider & Steenbock, 1939; Day & McCollum, 1939; Boelter & Greenberg, 1941). On the other hand, many reports on feeding experiments using the Steenbock diet make no mention of any paresis (Bachmann, Haldi, Wynn & Ensor, 1940; Zucker, Hall, & Young, 1941; Irving, 1944; Dodds & Cameron, 1943).

It was intended to investigate in turn the effects of supplements of vitamin E, of vitamin C and of the vitamin B group with a view to preventing the paralysis. When it was found that addition of vitamin E plus vitamin A in Exp. III did not prevent the paralysis, two additional experiments were set up. In Exp. IV the basal diet consisted of 1 part egg albumin mixed with 9 parts Steenbock diet; in addition, one-half of the rats received 5 mg. ascorbic acid every fifth day. Of the rachitic animals (group R) with no vitamin C, five out of six were in poor general condition at the end of four weeks, three had complete paralysis of the hindlegs and two showed paresis. On the other hand, rachitic (group R) animals which received vitamin C were in good condition

at the end of 4 weeks and none showed paralysis or paresis. Ten days later they were still in good condition and only two out of five showed paresis. Hence, addition of egg albumin to improve the quality of the protein gave no protection against paralysis of the hindlegs, but addition of vitamin C brought about great improvement in the general condition with at least partial protection against paralysis. Unfortunately, no experiments were carried out with Steenbock diet supplemented with vitamin C only.

A further experiment (V) was set up mainly to provide material for X-ray crystallography. The diet was the same as in Exp. III, but the maize was from a different source and no vitamin E was given. The average weight of the rats at the beginning of the experiment was 59 g.; this is a little higher than the starting weight in Exp. III. There was no paralysis in any of the seven rachitic rats at the time of killing, which was from 4 to 6 weeks from the beginning of the experiment. In all seven animals, the radiographic evidence of rickets was as clear as in any of the previous experiments. Exp. V suggests that there is some connexion between the original supply of maize and the occurrence of paralysis.

With the exhaustion of the original supply of maize went the possibility of further study of the problem of the paralysis. Our conclusions can therefore go only a little further than those of Shapiro and may be stated as follows. Paralysis was prevented by either vitamin D or vitamin C plus egg albumin (partially at least). Neither vitamins E plus A, nor egg albumin without vitamin C, was effective. The maize meal may have been either toxic or deficient in some respect and thus may have produced the paralysis.

One slight qualification to these conclusions should be mentioned. O'Brien & Morgareidge (1938) fed rats for 34 weeks on Steenbock diet alone without producing paralysis; the initial weight of their animals was about 175 g. This may only demonstrate further the variations in the quality of maize (see Jones, 1939), but such a long experiment would be likely to show up toxic effects if these existed. It may be that the paralytic signs appear only in an immature nervous system. The initial weight of our animals was very much less than in the experiments just quoted.

X-ray crystallographic results

General structure of bone. The extensive literature on the subject of bone structure has been reviewed recently by Fankuchen (1945) and the results of previous investigations on the structure of normal and pathological bone discussed. Although this present work was directed primarily towards a comparison of the structures of normal and of rachitic bone in rats, certain of the results obtained are of interest in connexion with the general problem of bone structure and will be dealt with first.

Dallemagne & Brasseur (1942*a*, *b*) and Brasseur, Dallemagne & Melon (1946) believe that the inorganic salt of bone consists of a mixture of α-tricalcium phosphate and calcium carbonate, which on calcination gives carbonato-apatite. After deproteinizing the bones by boiling with 6 % potassium glyceride these authors found that the diffraction pattern was very similar to that given by intact bone. This finding was taken to show that deproteinization by this method produced no structural changes in the inorganic salt. It does not seem justifiable to base such a conclusion on a diffraction pattern so lacking in detail, especially when most of the apatite series of minerals give a similar diffraction pattern. Their findings on structure are valid for the deproteinized material only and cannot be extended to the inorganic material as it occurs in intact bone.

In this investigation it has been found that, after the application of a milder process for the removal of protein, viz. trypsin digestion, a much more clearly defined diffraction pattern is obtained without variation in the spacings within the limits of measurement. Now a sharpening of this kind in the diffraction pattern given by a microcrystalline substance is due to an increase in the volume of the microcrystals having a completely regular structure, i.e. either a re-crystallization has taken place with the production of larger crystallites or there has been a removal of forces causing distortion of the crystallites in the intact specimen. The first conclusion is untenable both from a consideration of the insolubility of the inorganic salt and from the similarity of the degree of orientation shown by the inorganic crystallites in the intact deproteinized material. The conclusion must be that, on deproteinization by trypsin, the forces causing distortion of the inorganic crystallites are removed and the volume over which the structure is regular is increased. The forces involved are most probably those of attraction between protein and the surface areas of the inorganic crystallites, since it has been found that the orientation of both organic and inorganic material in bone is always closely similar in degree and direction. Further work is in progress in an effort to elucidate the nature of this organic-inorganic linkage.

Comparison of results obtained from normal and rachitic specimens of rat femora. Previous work in this field has been carried out by Clark & Mrgudich (1934) and by Reed & Reed (1942, 1945). In both investigations a disorienta-tion of the inorganic material in rachitic bones is reported, but only in the former paper is there any reference to the organic constituent.

Preliminary experiments with our material confirmed the following findings: (1) the orientation of the inorganic crystallites varies with the location in the bone of the specimen selected for examination (Reed & Reed, 1942), and (2) the degree of orientation shown by the specimen varies considerably with age (Caglioti & Gigante, 1936). It was established that these two factors have a similar influence on the organic constituent. The disturbing effects of these

factors were eliminated so far as possible from our experiments by selecting specimens from the same location in each bone examined and by making comparisons between animals of the same age.

No significant difference in orientation of either inorganic or organic material was found between normal and rachitic specimens. This result is at variance with that reported by Clark & Mrgudich (1934) and by Reed & Reed (1942, 1945). This difference cannot be ascribed to differences in technique since the methods used by us and by Reed & Reed are identical (personal communication). Analysis of Reed & Reed's published data shows that there is a significant difference between the mean ages of the animals whose bones showed poor orientation and good orientation; the older bones showed the better orientation. It is possible that the change in orientation reported by these workers is a natural change occurring with age rather than a response to antirachitic treatment. The fact that we do not find disorientation could be explained if the portion of the shaft used for diffraction studies had not been affected by the rachitic process. This is, however, difficult to reconcile with the very low ash content (c. 36%) of these bones as compared with the bones of the well fed animals (c. 60%).

This series of experiments has thus failed to give support to the idea of a peripheral action of vitamin D in the sense suggested by Reed & Reed (1945).

CONCLUSIONS

Our findings can be summed up most simply by stating that the deterioration in mechanical strength observed in rachitic bones is not associated with any chemical or structural alteration in the bone material. The strength of the bone material seems to depend entirely on the relative proportions of organic and inorganic material. This serves to emphasize our previous conclusion (Bell & Cuthbertson, 1943) that the organism has arrived at a well-defined and constant method of laying down bone. Neither calcium deficiency, nor hormone treatment, nor the rachitic disturbance seem able to disarrange this pattern so far as our methods are able to reveal it.

SUMMARY

1.   Male albino rats were fed from about 4 weeks to about 8 weeks of age on (a) Steenbock rachitogenic diet (group R). (b) this diet with addition of vitamin D (group N) and (c) an adequate diet (group S).

2.   The ash content of the femora in the three groups was: R, 36%; N, 43%; S, 60%. The Ca/P ratio was the same in all groups. The breaking stress on bending (a measure of the quality of the bone material) was about 12,000 lb. in group R, about 18,500 lb./sq.in. in group N, and about 27,000 lb./sq.in. in group S. There was a high correlation between ash content and breaking stress.

3. A large proportion of the R animals showed paralysis of the hindlimbs. This was not prevented by addition of vitamin A or of vitamin E or of albumin to the rachitogenic diet. Partial protection was afforded by addition of vitamin C and complete protection by vitamin D. On changing to a fresh stock of maize no paralysis was seen in the R animals.

4. The X-ray diffraction patterns obtained with intact bone, deproteinized bone, and decalcified bone are described. After a mild deproteinizing agent had been applied, the pattern given by the inorganic constituent became much more clearly defined than in the intact bone. This sharpening is presumably due to the release of force of attraction between the inorganic and organic material of the bone which produced a deformation of the inorganic crystals.

5. The diffraction pattern obtained from the rachitic bones (group R) is identical with that obtained from adequately fed animals (group S). There is no disorder of orientation of either inorganic or organic material in the rachitic bones. The degree of orientation of both organic and inorganic material is greatest at the centre of the shaft and diminishes to zero in the epiphysial area.

6. The results of all tests —mechanical, chemical and X-ray—suggest that there is no disturbance of the fundamental plan of ossification in rats suffering from rickets. The weakness of the rachitic bone is sufficiently explained by the reduction in inorganic material relative to the organic material.

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THE NORMAL VISUAL (ROD) FIELD OF
THE DARK-ADAPTED EYE

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The visual field of the completely dark-adapted eye, or rod field, was previously plotted for forty-seven presumably normal subjects using a 1.5×10^{-9} c.p. self-luminous radium-sulphide test object on a 330 mm. perimeter (Mann & Sharpley, 1946). The majority of the subjects were in the 10–20 and 20–30 years age groups. Thirty-three additional normal subjects, chiefly in older age groups, have now been similarly tested and the results are here presented for completeness.

METHODS

Apparatus and test procedure. The same apparatus and procedure were used as for the earlier tests already reported. Also, the method of expressing the size of the field is the same. Each subject was tested once only.

Subjects. The thirty-three subjects (seventeen males and sixteen females) were drawn from many different social levels and were all volunteers. They had no gross visual abnormalities, though many had moderate errors of refraction. No subject with a history of night-blindness was included. Otherwise no attempt was made to select cases.

RESULTS

The size of the field in terms of the mean angular radius for each subject tested is shown in Table 1. Average values (by age groups) are also given in the table for the whole group of eighty subjects comprised in the two series of tests (i.e. forty-seven in the earlier series and thirty-three in the present one) as one combined series. Fig. 1 shows frequency diagrams for each age group but one in the combined series.

Considering the results for the eighty subjects as a whole, two points of interest emerge. First, the spread of values within each age group is considerable and, secondly, there is an apparent tendency for the field to contract with increasing age. A reference to the first point has been made in the earlier paper dealing with the first results. It is now seen that the spread is not confined to the lower age groups and is even greater in the higher ones.

TABLE 1. Mean angular radius of rod field in degrees using radium-sulphide
test object of 1.5×10^{-9} c.p.

Subject	Sex	Age group (years)					
		10–20	20–30	30–40	40–50	50–60	60–70
1	F.	—	—	65	—	—	—
2	F.	—	—	64	—	—	—
3	F.	—	69	—	—	—	—
4	F.	—	—	58	—	—	—
5	M.	—	—	38	—	—	—
6	F.	—	74	—	—	—	—
7	F.	—	—	63	—	—	—
8	F.	—	59	—	—	—	—
9	F.	—	—	—	55	—	—
10	F.	—	60	—	—	—	—
11	F.	—	—	59	—	—	—
12	F.	—	—	61	—	—	—
13	F.	—	—	—	56	—	—
14	F.	—	—	68	—	—	—
15	F.	—	—	56	—	—	—
16	M.	—	—	—	54	—	—
17	M.	—	—	—	63	—	—
18	M.	—	—	—	—	58	—
19	M.	—	—	—	—	48	—
20	F.	68	—	—	—	—	—
21	F.	—	—	—	65	—	—
22	M.	—	—	—	57	—	—
23	M.	—	—	—	—	55	—
24	M.	—	—	—	64	—	—
25	M.	—	—	—	60	—	—
26	M.	—	—	—	57	—	—
27	M.	—	—	71	—	—	—
28	M.	—	—	—	—	45	—
29	M.	—	—	—	37	—	—
30	M.	—	—	51	—	—	—
31	M.	—	—	—	39	—	—
32	M.	—	—	—	56	—	—
33	M.	—	—	—	—	57	—
No. of subjects in each age group		1	4	11	12	5	—
No. of subjects previously tested		23	11	4	3	5	1
Total no. of subjects in both series of tests		24	15	15	15	10	1
Average mean radius of field for all subjects in both series		60°	63°	59°	52°	53°	40°

It is interesting to consider what factors may contribute to the cause of the shrinkage of the field with age. In the first place, there is one possible factor which is peculiar to the method of test. In general, older subjects, owing to greater muscular stiffness, cannot so freely turn the head to avoid physical restriction of the field by the facial features while keeping the eye fixated. This is particularly so for the extreme upper part of the field (i.e. the lower part of the retina) for which the head was flexed back to avoid interference by the eyebrows. This relatively greater stiffness was often noticed. It is not, however, considered that the results were very greatly affected on this account,

since, generally, older subjects showed a smaller field even on the lower temporal meridians where obstruction by facial features is negligible or non-existent.

It is also possible that older subjects found the plotting of the field under the somewhat difficult conditions to be rather more fatiguing than did the
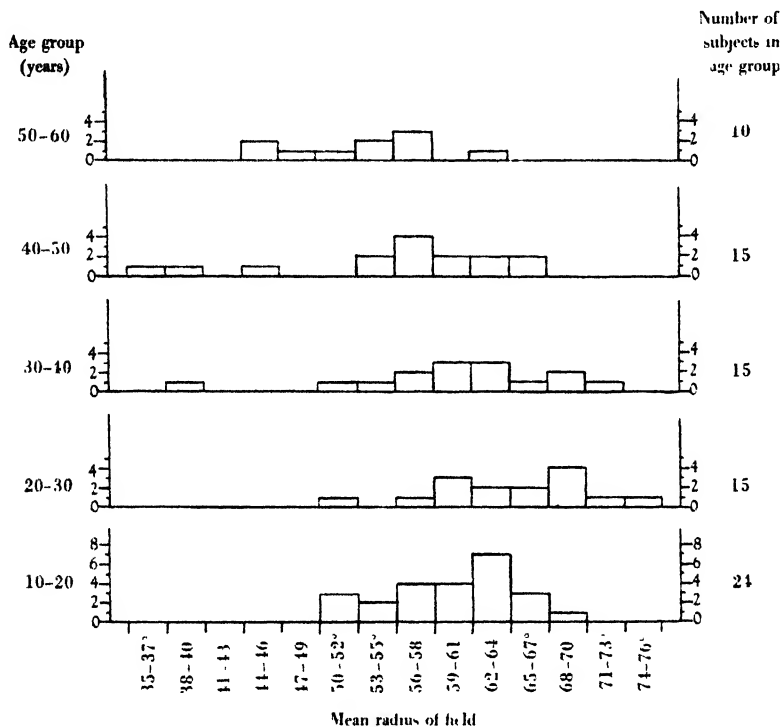
Fig. 1. Frequency diagrams showing mean radius of rod field for seventy-nine subjects by age groups.

younger subjects, and that they may not have concentrated their attention so well for this reason. But here again, any resulting effect is likely to be small since the method of test tends to eliminate reaction times.

The principal factors are probably mainly identical with those operative in raising the light threshold of the dark-adapted eye (as measured on one of the various forms of dark-adaptometer, using a stationary test-light of variable brightness). Such factors, which include yellowing of the lens, decrease of pupil diameter, retinal change, etc., have been discussed by others, e.g. Phillips (1939), Hecht & Mandelbaum (1939), and Robertson & Yudkin (1944). There is no published evidence to show that the light sensitivity of the periphery of the retina is affected by age relatively more than that of the more central areas within which most work on thresholds has been done.

It is well known that senile cystic degeneration affects the far periphery in the region of the ora serrata in old age and occurs in eyes otherwise normal. This may possibly have had some effect on the results, though it is considered doubtful for two reasons. In the first place, the present results appear to show a contraction of the field commencing in middle life. Secondly, even the largest fields measured are not as large as a number measured for the light-adapted eye in daylight with a 1° white test object by an otherwise similar technique. This shows that the outer physical boundary of the retina was not reached in the present tests.

It should, however, be recorded that in the course of the present tests it was observed that older subjects more often showed evidence of absolute or relative scotomata in the mid-periphery, or farther out (but still within the field boundary) due, presumably, to some degeneration of the retinal receptors (rods) with age. Examination with the ophthalmoscope seldom showed anything to note. An area of retinal insensitivity which would produce a scotoma or 'hole' in the visual field, if occurring not too far out in the periphery, could obviously be the cause of an indentation of the boundary of the field if farther out, and hence a contraction of the mean radius as measured to the boundary. It is probable, therefore, that the reduction of field size with age was partly accounted for in this way.

SUMMARY

1. The rod visual field of the completely dark-adapted eye was mapped out within 90° limits from the fixation point for thirty-three normal individuals.

2. The mean angular radius of the plotted fields for eighty subjects (including forty-seven previously tested) is given by age groups, and a tendency for the field to contract with advancing age is observed.

3. The possible reasons for this contraction with age are discussed.

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THE MEMBRANE RESISTANCE OF A
NON-MEDULLATED NERVE FIBRE

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Eight years ago, Cole & Curtis (1939) showed that the membrane resistance of the squid giant axon underwent a transient decrease during the passage of a nervous impulse. At about the same time Cole & Hodgkin (1939) obtained an approximate measurement of the membrane resistance in a resting axon. A comparison of the two sets of measurements showed that the membrane resistance at the height of activity was only about one-fortieth of that in the resting nerve. The large size of the resistance change suggests that other phases of nervous activity might be illuminated by measurements of membrane resistance. It would, for instance, be interesting to know what happens to the resistance during the refractory period or to discover how it is affected by ions and other chemical agents known to affect nervous activity. The second of these problems has been the main subject of my research, and the present paper contains an account of experiments which form an essential preliminary to this work. Previous determinations of membrane resistance have been made on the squid giant axon (Cole & Hodgkin, 1939; Cole & Baker, 1941*b*) and on the large axons of the lobster (Hodgkin & Rushton, 1946). Neither of these preparations is entirely suitable for a prolonged investigation, since the experimental animals are difficult to obtain and the axons must be dissected with great care before accurate measurements can be made. An attempt was therefore made to use the isolated axons of the shore crab *Carcinus maenas* (Hodgkin, 1938). These animals can be obtained throughout the year, and clean axons can be isolated from them without great difficulty. The membrane resistance of an isolated axon is so high that it can be measured with fair accuracy in spite of the small fibre diameter. The axon possesses the further advantage that its membrane resistance remains reasonably constant over a considerable period of time. Before starting to examine the effect of ions upon the membrane resistance it was necessary to settle two preliminary questions. First: what is the approximate value of the membrane resistance

in a normal axon? Second: what is the largest current which can safely be used for measurement without causing the membrane resistance to depart from its resting value? This paper attempts to answer these two questions and gives some additional information about other physical constants in *Carcinus* axons.

The method of estimating the membrane resistance is based upon that developed by Hodgkin & Rushton (1946) and depends upon the following principles. When a voltage is applied to a nerve fibre some of the current spreads along the fibre forming a local circuit in the extrapolar region. The distance over which this local circuit spreads in the steady state determines the spatial distribution of the extrapolar potential (electrotonus) and depends upon the resistances of nerve membrane, axis cylinder and external fluid. According to theory the potential should decline exponentially with distance and should fall to $1/e$ in a characteristic length which is related to the resistive constants in the following way:

$$\lambda = \sqrt{\frac{r_4}{r_1 + r_2}}. \tag{1}$$

λ is the characteristic distance or space constant, r_1 and r_2 are the resistances per unit length of the external fluid and axis cylinder respectively and r_4 is the resistance × unit length of the nerve membrane. The meaning of r_1 and r_2 is easily understood, but the dimensions and significance of r_4 may need further clarification. Suppose that the potential difference across the nerve membrane can be changed by a certain voltage over 1 unit length of nerve and that everywhere else the membrane potential has its normal resting value. Then the total current which flows across the membrane will be equal to the change in voltage divided by r_4. r_4 is expressed as a resistance × unit length because the total current which flows across the membrane increases with the length of nerve exposed to the applied voltage. r_2 and r_4 are fundamental constants for any particular axon, but their magnitudes depend upon the axon diameter. In comparing the properties of different axons it is therefore best to use the basic constants R_2 and R_4. R_2 is the resistance which would be observed if electrodes 1 cm. square could be placed on either side of a centimetre cube of axoplasm. R_4 is the resistance across 1 sq.cm. of membrane. In a cylindrical cell the two sets of constants are related in the following way:

$$R_2 = r_2 \pi a^2, \tag{2}$$

$$R_4 = r_4 2\pi a, \tag{3}$$

where a is the radius of the axon.

The determination of λ is the first step in estimating membrane resistance, but it must be followed by measurements of r_1 and r_2. The next stage consists in a measurement of the longitudinal resistance of the nerve under conditions which ensure that all the current is flowing parallel to the nerve membrane.

The resistance measured in this way is often called m and is defined by the relation

$$m = \frac{r_1 r_2}{r_1 + r_2}. \tag{4}$$

It may be determined by observing the potential gradient which exists between two electrodes separated by a large distance.

A third measurement must then be made in order to determine the relative magnitude of r_1 and r_2. No very satisfactory way of finding the ratio r_2/r_1 has been developed, but a reasonable estimate can be obtained by measuring the difference between the potential at a point where current is led into the nerve and a distant part of the extrapolar region. A necessary condition for measurement is that the anode and cathode of the applied current should be remote from one another. The voltage of the anode or cathode will be called V_A and is related to the applied current I in the following way:

$$\frac{V_A}{I} = \frac{m\lambda r_1}{2r_2}. \tag{5}$$

There are now three equations (1, 4 and 5) from which the three unknowns r_1, r_2 and r_4 can be determined.

When the membrane resistance has been measured it is a fairly simple matter to obtain the membrane capacity and this has been done in the present research. The membrane time constant is determined from the rate at which the extrapolar potential rises when current is applied and the membrane capacity obtained by the relation

$$C_m' = \tau_m / R_4, \tag{6}$$

where C_m' is the membrane capacity per sq.cm. and τ_m is the membrane time constant.

<div align="center">METHOD</div>

Five or six axons with a diameter of 25–35 μ. were isolated from one of the walking legs of *Carcinus maenas* by a method which has been described previously. Loose strands of connective tissue were detached from the axons with fine needles or knives. The cleanest and most uniform axon was mounted on the electrode assembly and raised into aerated paraffin oil. This method of recording gave satisfactory results, since axons were found to be capable of transmitting a large number of impulses and of surviving for 12–30 hr. Measurements were always made on excitable axons.

The electrodes consisted of glass tubes containing sea water and silver wires which had been coated electrolytically with chloride. One end of the tube was drawn into a capillary and terminated in a fine agar wick. The wick was made by allowing agar to solidify around a 60 μ. hair and had a thickness of about 100 μ. at the tip.

Electrical changes were recorded through cathode followers and a balanced d.c. amplifier similar to that described by Hodgkin & Rushton (1946). No grid leaks were employed and care was taken to ensure that stray leakage paths had a resistance greater than about 10^9 Ω. Rectangular pulses of current were applied to the nerve by an electronic circuit capable of producing pulses of variable width, amplitude and sign. One terminal of the pulse generator was earthed and was connected to electrodes A or C (Fig. 1). The other terminal was connected to the forceps F' through

an 0·5 μF. condenser and a resistance of 100 MΩ. The forceps behaved like a polarizable electrode, but the resulting polarization did not alter the form of the applied current since its effect was swamped by the series resistance of 100 MΩ. The current through the nerve was determined by inserting a monitoring resistance of 0·280 MΩ. and measuring the voltage across it. Reference should be made to a former paper (Hodgkin & Rushton, 1946) for a more detailed account of the circuit arrangements.

The absolute standard of resistance was a set of wire-wound resistors calibrated by the National Physical Laboratory, while the 50-cycle mains was used as the absolute standard of time.
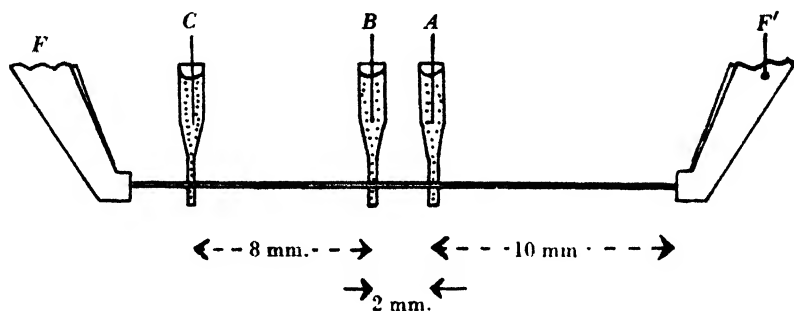
Fig. 1. Diagram of electrode arrangement. *A, B, C* are non-polarizable electrodes, and *F, F'* are metal forceps used for holding the axon.

PART 1

EXPERIMENTAL PROCEDURE

In previous measurements (Hodgkin & Rushton, 1946) the constants λ and m were determined from a number of observations made with a movable electrode. This was done partly in order to obtain the greatest possible accuracy and partly in order to check the validity of the theoretical equations. In the present work a simpler but less accurate method was employed. The nerve was arranged on the three agar wick electrodes shown in Fig. 1 and the distance between electrodes A and B carefully measured with a binocular microscope and an eyepiece micrometer. At the same time the effective thickness of each electrode was noted. The reason for using fixed electrodes was that the axons were subsequently needed for another investigation and 1 wished to avoid the technical difficulties associated with a movable electrode. The value of the space constant λ was obtained from the ratio of the membrane potentials at A and B when a weak positive current was applied between A and F'. The potential difference recorded between B and C (hereinafter called V_B) was directly proportional to the membrane potential at B. The potential recorded between A and C was spuriously increased by the voltage drop across the ohmic resistance of the agar wick and had to be corrected before a comparison with V_B could be made. Fortunately, the correction amounted to only about 5 % of V_A and an accurate evaluation was therefore unnecessary. An approximate estimate of the electrode resistance was obtained by dipping

the tip of the electrode into a large volume of sea water to a depth such that the oil-sea water interface coincided with the point where the axon normally made contact with the wick. The potential drop across the electrode was calculated from the current and the electrode resistance. The value so obtained was subtracted from the recorded potential difference and the resulting voltage (V_A) used for comparison with V_B. λ was determined by means of the equation

$$\lambda = l/\log_e (V_A/V_B), \tag{7}$$

where the length l was taken as the distance between the left-hand edges of electrodes A and B (Fig. 1). Some error was introduced by the fact that the electrodes were not infinitesimal, as assumed in the theory, but had a width of about 0·2 mm. However, this error should not have been large, since λ was about 2 mm. and the ratio of internal to external resistances was about 1·6.

The ratio V_A/I (eq. (5)) was determined by inserting a monitoring resistance of 0·280 MΩ. in series with electrode A and comparing V_A with the voltage drop across this resistance.

The parallel resistance of axis cylinder and external fluid (m) was determined by connecting the amplifier leads to electrodes A and B. and the pulse generating leads to C and F'. A comparison of the voltage recorded in this way with the voltage across the monitoring resistance gave the parallel resistance of axis cylinder and external fluid over the length AB. In calculating m this length was taken as the distance between the adjacent edges of the two electrodes.

At the end of each experiment the axon diameter was determined by microscopic observation. On the majority of occasions a binocular microscope with a magnification of 60 was employed. In certain cases these measurements were checked by observations with a $\frac{1}{8}$ in. water-immersion objective and an overall magnification of 800. On a number of occasions the axon diameter varied by as much as $\pm$ 20 %, and this variation must be regarded as one of the principal sources of error. But no serious systematic error seems to have been introduced, since the results obtained on uniform axons were of the same general order as those obtained on irregular ones.

The membrane time constant τ_m was usually measured by analysing a photographic record of the potential obtained at the polarizing electrode (V_A). According to theory (Hodgkin & Rushton, 1946) the curve so obtained should have the form

$$(V_A)_t = (V_A)_{t=\infty}\ \mathrm{erf}\ [\surd(t/\tau_m)] \tag{8}$$

for the make of current, where t is time.

Time constants obtained in this way are usually slightly longer than the true membrane time constant since the electrode is not infinitesimal as assumed by theory. A better method, which was used in one experiment. is to determine τ_m from the voltage (V_B) recorded at some distance from the

polarizing electrode. In this case the more complicated equation derived by Hodgkin & Rushton must be employed, viz.

$$(V_B)_t \underset{X}{=} (V_B)_{t=\infty} \underset{X=0}{\tfrac{1}{2}}\{e^{-X}\,[1 - \mathrm{erf}\,(X/2\sqrt{T} - \sqrt{T})] - e^{X}\,[1 - \mathrm{erf}\,(X/2\sqrt{T} + \sqrt{T})]\},$$

(9)

where $X = l/\lambda,\ T = t/\tau_m.$

Equation (8) can be applied very simply to the experimental results. A check is first made to ensure that the rise of potential agrees reasonably with the theoretical curve. The time constant is then taken as the time when the potential rises to 0·843 of its final amplitude.

RESULTS

The quantitative data obtained are summarized in Table 1. The measurements on which these results are based were obtained from experiments which were primarily directed towards another end. The data are therefore not as accurate as they might be and should eventually be replaced by more precise figures. But the results almost certainly give a correct order of magnitude for the physical constants in isolated *Carcinus* axons.

The average membrane resistance is about three times that found in the 70 μ. axons of *Homarus* and about ten times greater than that in the squid axon. The high values of membrane resistance encountered in the present work suggests that the density or mobility of the ions in the surface membrane must be extraordinarily low. The thickness of the surface membrane in *Carcinus* axons is likely to be of the same order as that in the mammalian red cell, since the membrane capacities are similar. According to Waugh & Schmitt (1940) the thickness of the lipoid layer in the red cell envelope is about 100 A. A layer of sea water 100 A. thick would have a resistance of only $2 \times 10^{-5}\ \Omega.$ cm.^{2} so that the product of ionic mobility and ionic density in an 8000 $\Omega.$ cm.^{2} membrane must be about $\tfrac{1}{4} \times 10^{-8}$ of that in sea water. There is nothing to show whether the high resistance of the cell membrane is primarily due to a low ionic density or to a low ionic mobility. A plausible compromise is to suppose that both are depressed to an equal extent. In this case the area of membrane containing one ion can be shown to be about $(\tfrac{1}{18}\,\mu.)^2$. This calculation suggests that the membrane must be a formidable ionic barrier. It also indicates that calculations about ionic movements in the membrane must be based on rather different premises from those used in dealing with the bulk phase of a solution.

Table 1 shows that the ratio of internal to external resistances is about 1·58. The action potential recorded from *Carcinus* axons averages about 45 mV. (cf. Hodgkin, 1938) so that the membrane action potential would be 45 mV. $\times (1 + 1\!\cdot\!58) = 116$ mV. This figure is of the same order as Hodgkin & Rushton's (1946) estimate for *Homarus* axon and as the direct measurements of Curtis & Cole (1942) or Hodgkin & Huxley (1939, 1945) on *Loligo* axons.

TABLE 1. Electrical constants in *Carcinus* axons

Axon diameter (μ.)	λ (mm.)	τ_m (msec.)	r_2/r_1	R_2 (Ω. cm.)	R_4 (Ω. cm.²)	C_m (μF. cm.⁻²)
33·9	2·04	5·96	1·34	98·9	8490	0·702
35·6	2·82	—	1·52	88·4	13050	—
{34·8	2·46	8·17	1·55	80·6	9190	0·889
{34·8	2·28	8·73	1·47	84·7	8500	1·027
{30·5	1·64	—	1·58	70·9	4080	—
{30·5	1·35	4·60	1·15	67·1	3000	1·531
[27·8	1·66	4·40	1·75	93·8	5830	0·754
[27·8	0·89	4·15	1·59	109·3	2050	2·025
{30·9	2·67	9·75	2·20	115·8	15600	0·625
{30·9	1·68	8·60	1·75	111·5	6420	1·340
32·2	2·34	—	1·44	69·2	7970	—
Average						
31·8	1·98	6·79	1·58	90·0	7653	1·112

Square brackets indicate that the two sets of measurements were made on the same nerve fibre; curved brackets that they were made on the same stretch of the same nerve fibre. An interval of several hours elapsed between the two sets of measurements. Data obtained over period April to August 1946. Temperature 15–18° C.

Another interesting conclusion can be drawn from Table 1. According to mathematical theories, such as those of Offner, Weinberg & Young (1940), the velocity of propagation should decrease by the factor $\sqrt{r_2/(r_1+r_2)}$ when the axon is removed from a large volume of sea water in which $r_2 \gg r_1$ and immersed in oil in which r_2 is comparable to r_1. The basis of Offner, Weinberg & Young's calculation may be regarded as speculative, but it can be shown that the square-root law is completely general and makes no assumptions about the membrane beyond the fact that it is capable of transmitting an impulse by local circuit action at constant velocity (unpublished calculations). In *Carcinus* axons the ratio (velocity in sea water/velocity in oil) was found to have a mean value of 1·27 and the standard deviation of the mean of sixteen observations was 0·018 (Hodgkin, 1939). Table 1 indicates that the quantity $\sqrt{(r_1+r_2)/r_2}$ has a mean of 1·28 and a standard deviation of the mean of 0·012 (eleven observations). There is therefore a close agreement between the quantitative predictions of the local circuit theory and the results of two quite different sets of observations. As Offner *et al.* (1940) have pointed out, Cole & Hodgkin's (1939) data are also in reasonable agreement with Hodgkin's (1939) measurement of the conduction velocity change in *Loligo* axons.

The individual values for the axoplasm resistivity must be regarded with some suspicion, since they were each based on a single measurement and not on a set of points (cf. Hodgkin & Rushton, 1946). The calculated values were also subject to the errors introduced by non-uniformity of axon diameter and by the assumption of infinitesimal electrode width. But the average result obtained is not very different from that found in other animal cells which are known to have a resistivity several times greater than that of the external medium.

Table 1 indicates that the membrane resistance decreased and the capacity increased when successive measurements were made on the same axon. The decrease in membrane resistance has been observed before (Cole & Hodgkin, 1939; Hodgkin & Rushton, 1946) and is probably due to some kind of progressive deterioration of the surface membrane. The increase in membrane capacity was not observed by Hodgkin & Rushton and cannot be regarded as established by the three experiments given in Table 1.

PART 2

The values for membrane resistance shown in Table 1 were obtained with anodic currents with strengths of the order of 1-2 × threshold. The experiments to be described in this section were made in order to discover whether such currents had any disturbing influence upon the membrane resistance. In other words, their object was to find the range of current over which the nerve membrane obeys Ohm's law.
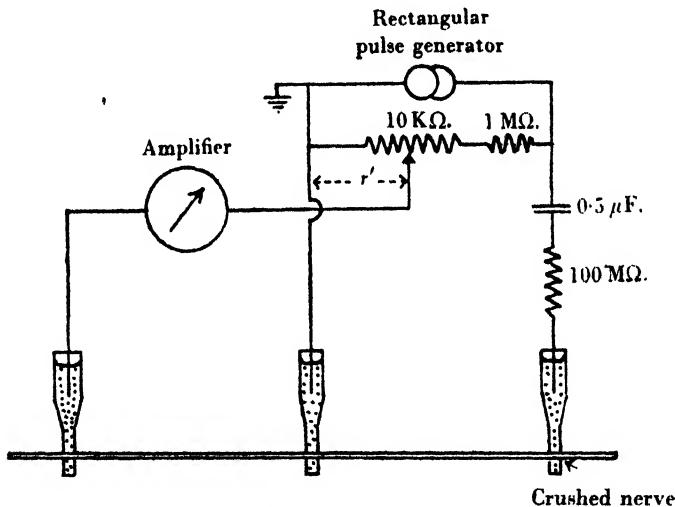
Fig. 2. Diagram of circuit used for measuring effect of current on membrane resistance.

The axon was arranged in the manner shown by Fig. 2, and rectangular waves of current with a duration of about 200 msec. were applied from the pulse generator. When the potentiometer slider was moved to the extreme left ($r' = 0$) the potential recorded was that given by the sum of the membrane potential and the small potential resulting from the ohmic resistance of the electrode. The steady potential which was established after about 10 msec. could be neutralized by moving the potentiometer slider to the right. Balance was achieved when $R_4 = k_1 (k_2 r' - \Delta)^2$. In this equation Δ is the electrode resistance, while k_1 and k_2 are factors which do not depend upon membrane resistance and so do not vary with current. It was therefore possible to study

the effect of current on membrane resistance by determining the balance position for different strengths of current. A typical experiment is illustrated by Fig. 3, in which relative membrane resistance is plotted against current. The results show that the membrane resistance was nearly constant over a wide range of anodic currents, but appeared to increase markedly when the current was cathodic and approached threshold. In this particular experiment there was also a small increase of resistance on the anodic side, but it is clear that any strength of current up to about three times threshold may be employed without introducing serious error.
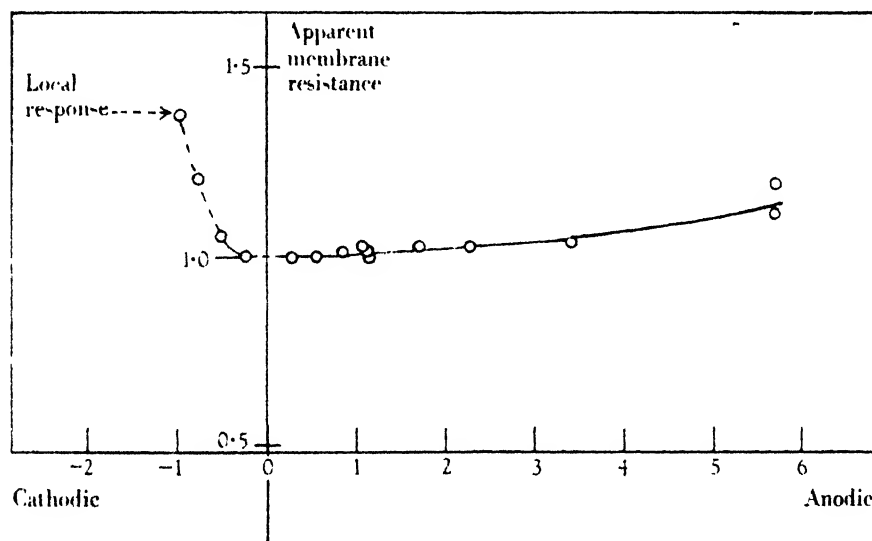
Fig. 3. Ordinate: apparent value of membrane resistance. 1 unit $\doteqdot$ 8000 Ω. cm.^{2} Abscissa: current through axon in units such that -1 = threshold current (rheobase). 1 unit $\equiv$ 1·30 × 10^{-8} amp. through axon $\equiv c$. 1·14 × 10^{-6} amp.cm.$^{-2}$ membrane current density under electrode.

The apparent increase of resistance on the cathodic side seems to conflict with Cole & Baker's (1941 a) observation that the membrane resistance decreases under the cathode. The contradiction can be resolved in the following way. The increase in resistance observed with a just subthreshold current was definitely due to a local response. Instead of rising to a steady maximum the membrane potential showed a prolonged but definite hump (cf. Hodgkin & Rushton, 1946, fig. 15). The flat maximum of this hump was balanced in a bridge so giving the value of 1·4 shown in Fig. 3. At 0·5 threshold there was no definite hump and the potential appeared to rise to a steady maximum. But there was complete continuity between the two sets of curves, and it is not difficult to believe that a 0·5 threshold current evoked a very slight but sustained response which added to the passive electrotonic potential and appeared to increase the membrane resistance. Cole & Baker (1941 a)

determined the membrane resistance change with transverse electrodes and alternating current of frequency about 5 kcyc./sec. Under these conditions a resistance decrease might be expected, since the steady e.m.f. changes associated with subthreshold activity would not affect the a.c. bridge measurements, and the only change observable would be the actual decrease in resistance associated with subthreshold activity.

Cole & Curtis (1941) studied the membrane voltage-current relation with direct current applied to impaled axons and again found a decrease in resistance when strong cathodic currents were employed. But this experiment was made under very different conditions from mine. In the first place the small size of the membrane action potential (< 50 mV.) and the extremely
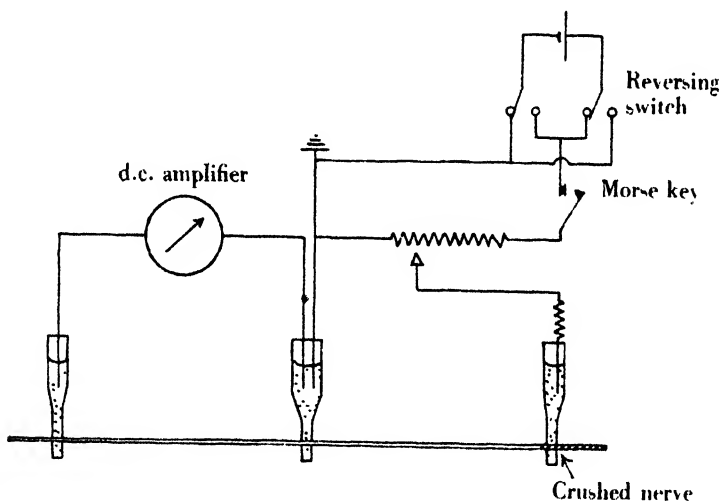
Fig. 4. Arrangement for determining current-voltage relation with currents of long duration. The electrodes are of the silver chloride-agar-wick type.

low membrane resistance (23 Ω. cm.²) suggest that the measurements were made on nerve which had been depolarized by proximity to the point where the micro-needle punctured the axon. Furthermore, the actual membrane current densities used by Cole & Curtis were about 300 times greater than those employed in my experiments, so that no physical comparison of the two results can really be made. The effect observed by Cole & Curtis can be seen in *Carcinus* axons when the current is increased beyond the point at which impulses arise and is left on for a long time. In this case a prolonged discharge of impulses occurs, but the membrane potential eventually attains a steady level which bears the same kind of relation to current as that found by Cole & Curtis. This point is illustrated by an experiment in which the voltage-current relation was determined with a wide range of currents lasting several seconds. A double electrode was employed in order to remove any possibility of electrode

polarization, and electronic complications were avoided by using a simple circuit operated by a morse key (Fig. 4). The results are shown in Fig. 5; curve 1 indicates the steady voltage finally attained, while curve 2 shows the
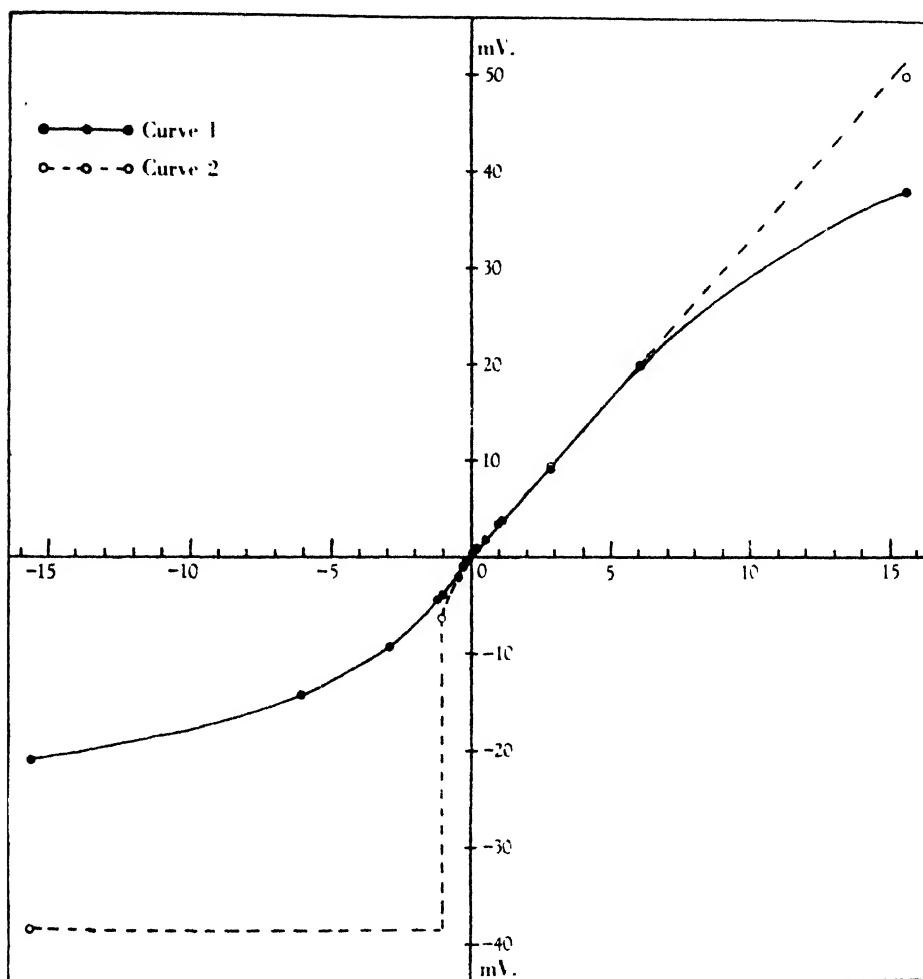
Fig. 5. Ordinate: change in potential produced by current. Curve 1 steady deflexion; curve 2 extreme deflexion. Abscissa: current through axon expressed in units such that -1 = threshold. 1 unit $\doteqdot 0.866 \times 10^{-8}$ amp. $\doteqdot 1.52 \times 10^{-6}$ amp.cm.$^{-2}$ membrane current density under electrode (rough estimate only).

extreme voltage evoked by each current. On the anodic side the nerve behaved in a relatively simple manner. The voltage was proportional to current over most of the range, and the potential time curves rose to their maximum without overshoot. The absence of overshoot is indicated by the coincidence of curves 1 and 2 and by the typical record of Fig. 6d. The slight increase

in resistance shown in Fig. 3 was either absent or not revealed by the relatively insensitive method of measurement. With a very strong anodic current, the potential was not maintained but declined from its original maximum. One way of explaining this effect would be to assume that the membrane was temporarily damaged by the large voltage across it. This hypothesis received support from the fact that anodic pulses of this magnitude were followed by a long burst of impulses (Fig. 6*e*). The results were more complicated when
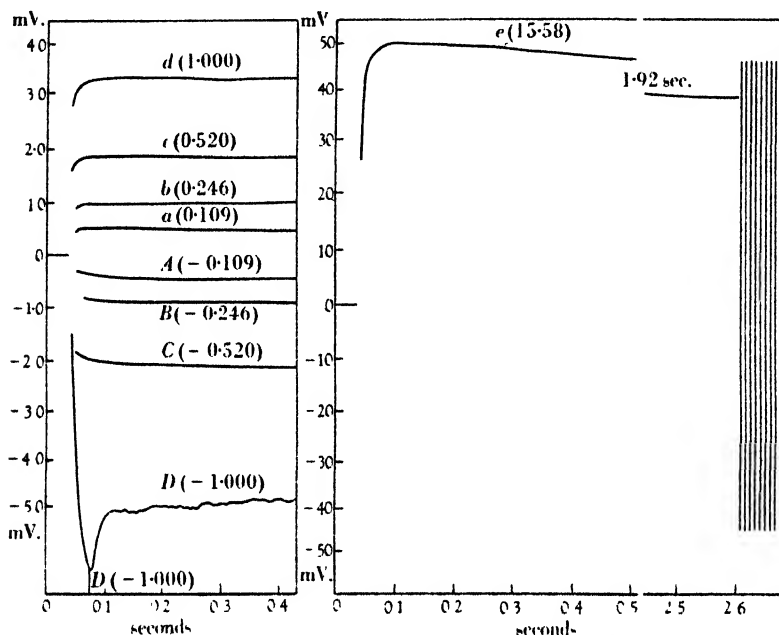
Fig. 6. Typical voltage-time curves used in plotting Fig. 5. Current strengths given in units such that $-1 =$ threshold. In *e* the action potentials which follow the break of current are shown diagrammatically.

cathodic currents were employed. With very weak currents the voltage was proportional to current and no overshoot occurred. Just below threshold a prolonged local response was produced. Since this showed a definite hump the curves for extreme and steady potential diverged. The divergence was greatly accentuated at threshold because curve 1 then fell to the extreme level determined by the action potential while curve 2 started to bend in the opposite direction. Currents appreciably greater than threshold evoked a long train of impulses and a steady potential was not attained for several seconds. But the steady potential, which was eventually produced, followed the same general type of curve as that described by Cole & Curtis (1941). When the current was strong and cathodic the voltage-current gradient was clearly much less than that obtained with weak or anodic currents, and this effect may be interpreted as a decrease in membrane resistance.

Some of the voltage-time curves used in this experiment are shown in Fig. 6. In *a, b, c, d, A* and *B* the membrane behaved like a linear circuit element containing only resistance and capacity. In *C* there was no overshoot, but the slight difference between anodic and cathodic curves suggests the presence of subthreshold activity. *D* shows a 'humped' local response with a duration of about 50 msec. The fact that the cathodic potential exceeded the corresponding anodic potential throughout the entire period of current flow suggests that the axon was maintained in a state of subthreshold activity after the end of the humped local response.

The complicated effects shown in Figs. 3, 5 and 6 are not claimed to be a fundamental property of all excitable membranes. Nor is it certain that they represent the behaviour of *Carcinus* axons over a wide range of experimental conditions. But one quite definite fact emerges from experiments of this type. Namely, that accurate measurements of membrane resistance in *Carcinus* can only be obtained when the current is anodic or less than about one-third threshold. This fact was not clearly recognized in the past, and neglect of it may have introduced occasional errors. Cole & Hodgkin (1939) used both cathodic and anodic currents in their determination of the resistance-length curve in squid axons. But the strength of current employed was only one-tenth threshold and should therefore have given satisfactory results. Hodgkin & Rushton (1946) used cathodic currents of strength 0·4 to 0·5 threshold so that some of their results may have been complicated by traces of local activity. But the errors introduced in this way could not have been large, since the determination of λ was based on measurements which extended far into the extrapolar region where the membrane current was much less than one-third threshold. In the present work cathodic currents were never employed and the anodic currents used were too weak to have any appreciable effect on the membrane resistance.

SUMMARY

1. The electrical constants of isolated axons from *Carcinus maenas* were measured with pulses of direct current and longitudinal electrodes.

2. The electrical resistance of the nerve membrane varied between 2000 and 16,000 Ω. cm.^{2} in excitable axons and had an average value of about 8000 Ω. cm.^{2}

3. The average capacity of the surface membrane was 1·1 μF. cm.$^{-2}$

4. The specific resistance of the axoplasm was found to be about 90 Ω. cm. (4 times sea water).

5. The ratio of internal to external resistance per unit length was approximately 1·6.

6. The nerve membrane was found to obey Ohm's law over a wide range of anodic currents, but showed marked deviations with relatively weak cathodic currents.

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THE EFFECT OF POTASSIUM ON THE SURFACE MEMBRANE OF AN ISOLATED AXON

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The object of this paper is to examine the effect of potassium and other monovalent cations on the electrical resistance of the surface membrane in a non-medullated nerve fibre. This problem is interesting because potassium ions are known to have a pronounced effect on many phases of nervous activity, and it is possible that some of these effects are connected with changes in membrane resistance. There are, in fact, good reasons for believing that the electrical resistance of the surface membrane should be altered by small changes in the potassium content of the external medium. According to the membrane theory of nervous action, the surface of a nerve or muscle fibre is permeable to potassium but relatively impermeable to sodium ions. If the theory is correct it follows that potassium ions must be responsible for carrying a large part of the current which flows through the membrane when a potential difference is applied. An increase in the external potassium concentration should be followed by a similar change in the outer layers of the membrane and should therefore cause a decrease in membrane resistance. Qualitative effects of this kind have been observed in *Nitella* by Blinks (1929) and Osterhout (1931), while Cole & Marmont (1942) have given a preliminary account of the effect of potassium upon the membrane resistance in the giant axon of the squid. The aim of the present paper is to describe the quantitative relation between the external potassium concentration and the membrane resistance of isolated axons of *Carcinus maenas*.

Methods of determining membrane resistance in isolated axons have been described by Cole & Hodgkin (1939), Hodgkin & Rushton (1946) and Hodgkin (1947). The results established the validity of the theoretical equations and gave the absolute magnitudes of the membrane resistance in three different preparations. But none of these methods lends itself very readily to an investigation of the effect of changes in the ionic content of the external fluid. The greater part of the axon must be immersed in oil and is therefore surrounded only by a thin film of salt solution. The composition of this film could be changed by dipping the nerve fibre into different solutions, but there would be no way of ensuring that the saline film retained the composition of the solution

to be tested. The volume of external fluid which clings to an axon in oil is small compared to the volume of axoplasm, and its composition would change rapidly if ions enter or leave the axis cylinder. The effect of ions on membrane resistance should therefore be determined on a stretch of nerve which is immersed in a large volume of test solution. It is also desirable that the composition of the test solution should be capable of being changed rapidly and with little mechanical disturbance to the axon. A method which satisfies these two criteria has been developed, and the results obtained with it are described in the first part of this paper. The second part is concerned with observations made by the 'dipping' method and shows that potassium ions may be absorbed rapidly from the external fluid.

METHOD

The apparatus used is shown diagrammatically in Text-fig. 1, and was similar in most respects to that employed in previous work. A novel feature is the arrangement used for applying test solutions. This consisted of a thin-walled glass tube about 2 mm. in diameter and 20 mm. in length which was mounted rigidly on the electrode assembly. Two small holes were made in the walls of this tube by means of a microflame and a fine platinum wire. The position of the holes was such that the nerve fibre could be drawn transversely through the tube and mounted in the position shown in Text-fig. 1. The solution in the tube could be changed by adding 1 c.c. of test fluid to the top of the tube with a fine pipette or hypodermic syringe. This operation washed the tube free from its original solution and left it filled with the solution to be tested. Diffusion and convection at the bottom of the tube must have slowly altered the composition of the solution, but tests with coloured fluids showed that the solution in contact with the axon remained unaltered for several hours. In certain experiments the possibility of convection was removed by lowering the tube on to a pad made from filter paper (G).

The following procedure was used for mounting the nerve in the correct position. The tube was lowered to a depth such that the two radial holes were immersed in Ringer's solution. A $60\,\mu$ hair was threaded through the two holes and a noose tied at the end of the hair. The axon was caught in this noose and pulled across the tube by the hair. The ends of the axon were gripped in the forceps and the hair removed with scissors. The whole assembly was then raised into oil and the electrodes D and C brought into contact with the axon. In this condition the tube was filled with Ringer's solution and the axon was in position for the first electrical measurements. The operation of mounting the nerve fibre required a certain amount of practice and axons were often damaged by clumsiness or accidents, but the equipment itself did not seem to have any deleterious effect on the viability of the isolated axon.

Rectangular pulses of current with a duration of about 50 msec. were applied to the nerve through the forceps F' and the non-polarizable electrode E. Current flowed into the end of the axon and returned through the solution in the tube to the electrode E. The polarity of the current was such that the forceps were cathodic and the tube anodic. The polarization at the tip of the forceps did not alter the form of the applied current, since the effect of this polarization was swamped by the series resistance of 10–20 MΩ.

Some of the current applied to the nerve flowed into the extrapolar region forming a local circuit. A small potential was therefore recorded when the amplifier was connected to the points B and C. This potential was a constant fraction of the membrane potential change at the distal edge of the tube and will be called V_b. Its magnitude varied with the membrane resistance of the nerve exposed to the test solution, but measurements of V_b alone could not give more than a qualitative indication of changes in membrane resistance. The results could be made quantitative by recording the potential difference between the tube and electrode D. The voltage recorded in

this way consisted of the voltage drop across the ohmic resistances of axis cylinder and external fluid as well as that across the membrane. The part of the voltage depending on the membrane will be called V_a. The potential drop across the ohmic resistances was balanced out by means of the bridge potentiometer P.
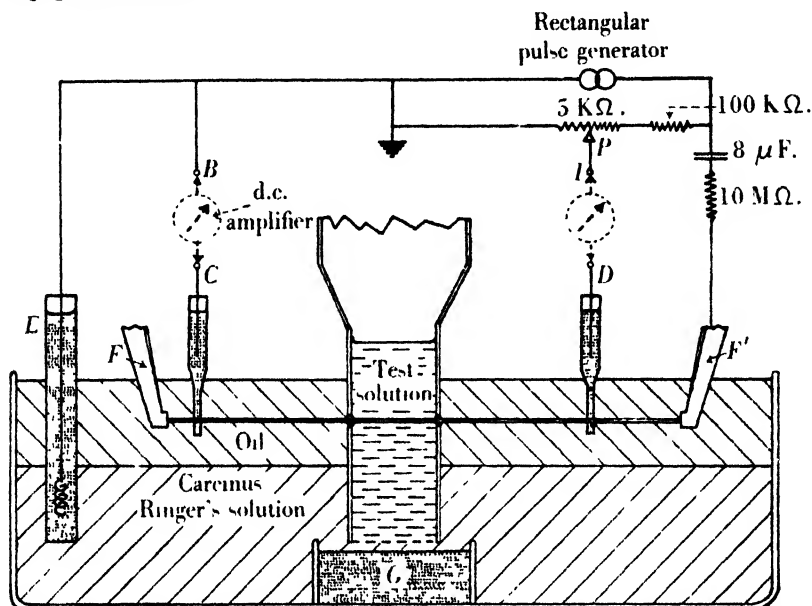
Text-fig. 1. Diagram of apparatus used for testing effect of ions upon membrane conductance. E, C and D are non-polarizable electrodes of the silver-silver chloride type. See text for other letter references.

All measurements were made by comparing the membrane resistance of a section of axon exposed to a test solution with that of the same section in contact with a normal solution. Sea water was occasionally used as a normal solution, but a special *Carcinus* Ringer's solution was employed in the greater part of the work. This solution was made according to Pantin's (1946) formula and is based on Webb's (1940) analysis of *Carcinus* blood. Its composition was as follows:

Na$^+$	493·6 mm.	Cl$^-$	535·2 mm.
K$^+$	11·3 mm.	SO$_4^{--}$	15·4 mm.
Ca^{++}	12·3 mm.	HCO$_3^-$	0·2 mm.
Mg^{++}	18·3 mm.		

Figures for the concentration of the principal ions in sea water are given for comparison. They are based on the data provided by Harvey (1945) and a salinity of 3·46 %:

Na$^+$	456 mm.	Cl$^-$	537 mm.
K$^+$	9·8 mm.	SO$_4^{--}$	27·5 mm.
Ca^{++}	10·0 mm.		
Mg^{++}	52·5 mm.		

The potassium content of the Ringer's solution was usually changed by adding (or subtracting) a definite weight of potassium chloride. This procedure was adopted because it allowed a direct comparison to be made between the addition of x mol. of potassium chloride and y mol. of sodium chloride. Such a comparison would have been impossible if the method of mixing isotonic solutions had been employed. The procedure is open to the objection that the abnormal tonicity of the solutions employed might have introduced spurious effects. But the change in tonicity was extremely small and cannot be regarded as abnormal, since the tonicity of *Carcinus* blood varies

over wide limits in the natural state (Schlieper, 1929). Moreover, the nerve fibre is probably not impermeable to potassium chloride (cf. Boyle & Conway, 1941; Shanes 1946), so that from some points of view it is better to add solid KCl than an isotonic solution. The argument is in any case of little importance, since both methods of adding KCl gave essentially the same result.

Rubidium, caesium and lithium chlorides were used in certain experiments. All three salts were obtained from reliable sources, but no figures could be obtained for the purity of the rubidium and caesium salts. The results obtained were therefore checked with solutions made from spectro-scopically purified salts. The purity of these compounds was given as 99·9995%.

THEORETICAL SECTION

The purpose of this section is to show how the effects of ions upon the conductance (or resistance) of the surface membrane can be calculated from observations with external electrodes. The method of analysis is essentially similar to that described by Hodgkin & Rushton (1946) or Cole & Hodgkin (1939), and will therefore be given in as brief a manner as possible. The nerve fibre is assumed to have a uniform cable-like structure with a conducting core surrounded by a membrane, the resistance of which obeys Ohm's law for small current densities. It is further assumed that the solutions applied did not change the electrical resistance of the axis cylinder. This simplification was justified by the fact that the change in membrane conductance was complete within a few seconds, so that measurements were made before any appreciable diffusion into the axis cylinder could take place.

The geometry of the theoretical system considered is shown in Text-fig. 2 and the symbols employed are listed below. The term 'polar region' is used to describe the part of the nerve in the tube, since this region can be regarded as an equipotential electrode:

x is distance along axon.

i_1 is current in external fluid.

i_2 is current in axis cylinder.

I is total current flowing through the fibre and external fluid ($I = i_1 + i_2$).

V_1 is the potential of the external fluid with respect to a distant point

$$\left(V_1 = -\int_{-\infty}^{x} r_1 i_1 dx\right).$$

V_m is the change in potential difference across the surface membrane which results from the flow of current.

r_1 is the resistance per unit length of the external fluid which clings to the fibre in oil.

r_2 is the resistance per unit length of the axis cylinder.

g_4 is the conductance per unit length of the membrane in the normal nerve in Ω^{-1} cm.$^{-1}$ The reason for using membrane conductance instead of resistance in the present paper is that the action of potassium is described most easily in terms of conductance.

$\beta^2 g_4$ is the conductance per unit length of the membrane in the polar region $\beta^2 = 1$ when the polar region contains normal Ringer's solution.

λ_n is the space constant of the normal axon in oil and is equal to $[g_4 (r_1 + r_2)]^{-\frac{1}{2}}$.

λ_p is the space constant of the normal axon in the polar region and is equal to $[g_4 r_2]^{-\frac{1}{2}}$.

$\alpha \quad = \sqrt{\dfrac{r_1 + r_2}{r_2}} = \dfrac{\lambda_p}{\lambda_n}$.

$m \quad = \dfrac{r_1 r_2}{r_1 + r_2}$ and is the parallel resistance of core and external fluid.

l is the width of the tube.

$\theta \quad = l/\lambda_p$.
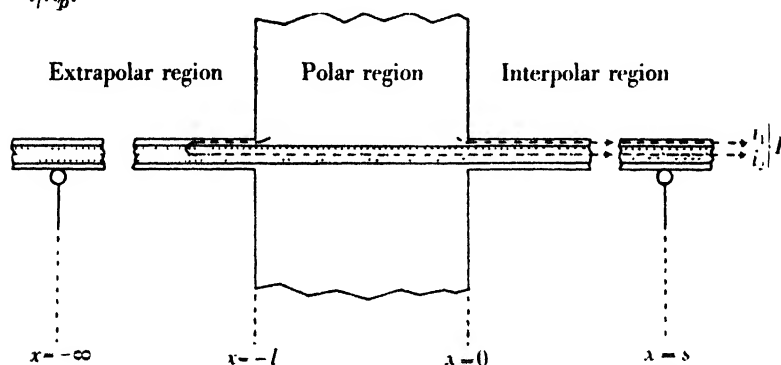
Text-fig. 2. Geometry of system considered in theoretical section.

A sharp boundary between the polar region and the interpolar or extrapolar regions is assumed and the tube is taken to extend from $x = 0$ to $x = -l$. The externally applied current I flows from $x = 0$ to $x = +\infty$. Recording electrodes are connected to the points $x = -\infty$, $x = 0$ and $x = s$, where $s \gg \lambda_n$.

The general equation for the response of a nerve fibre to a weak anodic current or a very weak cathodic one is

$$-\lambda^2 \frac{\partial^2 V_m}{\partial x^2} + \tau_m \frac{\partial V_m}{\partial t} + V_m = r_1 \frac{\lambda^2}{\partial x} \frac{\partial I}{} \tag{1}$$

(see Hodgkin & Rushton (1946), equation (2·1)). The term containing t can be omitted since a steady state is to be considered. A further simplification is that

$r_1 \dfrac{\partial I}{\partial x}$ vanishes except at the point $x = 0$. Hence

$$\lambda_n^2 \frac{d^2 V_m}{dx^2} = V_m, \quad \text{when} \quad -\infty < x < -l, \tag{2}$$

$$0 < x < \infty$$

and $\dfrac{\lambda_p^2}{\beta^2} \dfrac{d^2 V_m}{dx^2} = V_m, \quad \text{when} \quad -l < x < 0.$ $\tag{3}$

These equations must be solved for the conditions that $V_m = 0$ when $x = -\infty$ or $+s$ and that V_m and i_2 are continuous from $x = -\infty$ to $x = +s$. V_m and i_2 are related by the following equation (cf. Hodgkin & Rushton, 1946, equation (1·2)):

$$\frac{dV_m}{dx} = (r_1 + r_2)\, i_2 - Ir_1. \tag{4}$$

The following relations are obtained when the solutions of (2) and (3) are applied to the points $x = 0$ and $x = -l$:

$$(V_m)_{x=0} = r_1 \lambda_n I \left\{ \frac{(\alpha\beta + 1) + (\alpha\beta - 1)\, e^{-2\beta\theta}}{(\alpha\beta + 1)^2 - (\alpha\beta - 1)^2\, e^{-2\beta\theta}} \right\}, \tag{5}$$

$$\frac{(V_m)_{x=0}}{(V_m)_{x=-l}} = \frac{\sinh \beta\theta}{\alpha\beta} + \cosh \beta\theta. \tag{6}$$

These equations must now be related to observations of the external potential.

According to Hodgkin & Rushton (1946, equation (1·3))

$$V_m = \left(\frac{r_1 + r_2}{r_1}\right) V_1 + r_2 \int_{-\infty}^{x} I\, dx \tag{7}$$

In the extrapolar region I is zero so

$$(V_1)_{x=-l} = \left(\frac{r_1}{r_1 + r_2}\right)(V_m)_{x=-l}. \tag{8}$$

$(V_1)_{x=-l}$ is measured experimentally and is called V_b. In the interpolar region

$$(V_1)_{x=0} - (V_1)_{x=s} = \frac{r_1}{r_1 + r_2}\{(V_m)_{x=0} - (V_m)_{x=s}\} + m\int_0^s I\, dx. \tag{9}$$

If s is made sufficiently large $(V_m)_{x=s} = 0$ and

$$(V_1)_{x=0} - (V_1)_{x=s} = \frac{r_1}{r_1 + r_2}(V_m)_{x=0} + mIs. \tag{10}$$

When the current is suddenly applied to a nerve the membrane potential (V_m) does not change instantaneously because the membrane capacity short-circuits the membrane resistance. On the other hand, current flows instantaneously in the core and external fluid so that the voltage drop represented by the term mIs is established instantaneously. In my experiments the voltage difference mIs was balanced in a bridge and the quantity remaining (called V_a) was measured directly. V_a is formally defined by the relations ·

$$V_a = (V_1)_{x=0} - (V_1)_{x=s} - mIs = \frac{r_1}{r_1 + r_2}(V_m)_{x=0} \tag{11}$$

The following relations can therefore be derived from (5), (6), (9) and (11):

$$\frac{V_a}{V_b} = \frac{\sinh \beta\theta}{\alpha\beta} + \cosh \beta\theta, \tag{12}$$

$$\frac{V_a}{I} = \frac{m\,(\alpha^2 - 1)\, l\, [(\alpha\beta + 1) + (\alpha\beta - 1)\, e^{-2\beta\theta}]}{\alpha\theta\,[(\alpha\beta + 1)^2 - (\alpha\beta - 1)^2\, e^{-2\beta\theta}]}. \tag{13}$$

In a normal axon, where $\beta = 1$, equation (12) becomes

$$\frac{V_a}{V_b} = \frac{\sinh \theta}{\alpha} + \cosh \theta. \tag{15}$$

The exponential terms in (13) may be neglected, since $\beta = 1$, $\alpha \doteqdot 1\cdot3$ and $\theta \doteqdot 1$ in the normal axon. Equation (16), which is correct to 3 %, is then obtained:

$$\frac{V_a}{I} = \frac{m\,(\alpha - 1)\,l}{\alpha \theta}. \tag{16}$$

Equations (12), (15) and (16) were employed for calculating β. Measurements of m, V_a/I, V_b and l were first made in a normal axon and the values of α and θ determined by numerical solution of equations (15) and (16). A test fluid was run into the tube and a new value for the ratio V_a/V_b determined. A value for β was then obtained by equation (12). By definition, the square of this value gives the change in membrane conductance. Thus a value of 2 for β means that the membrane conductance has been quadrupled and the resistance reduced to one-quarter of its former value.

The correct value for m was determined by noting the distance s and the bridge setting needed to balance the voltage drop represented by the term mIs.

RESULTS

Part I

The results of a typical experiment are shown in Pl. 1. Record 1 b was obtained with normal Ringer's solution in the tube and the amplifier connected to points B and C (Text-fig. 1). The potential obtained in this way (V_b) is equivalent to an extrapolar potential recorded at a distance of about one space constant from the point where current is led into the nerve. It therefore shows traces of the S-shaped time course characteristic of all such records. Record 1 a was obtained from the same solution but with a lower amplification and with the amplifier leads connected to I and D. The sudden rise of potential resulting from the flow of current through the nerve has been balanced with the bridge arrangement of Text-fig. 1. The balance point could not be determined exactly, since the membrane started to charge at an infinite rate, so that there was no sharp distinction between the ohmic and capacitative processes. Fortunately, errors in the determination of the balance point were not important, since the properties of the theoretical equations are such that a large error in V_a introduces only a small change in β. The approximate balance point was obtained by increasing the time-base speed 10–100 times and noting the point at which the rate of rise first started to decline from its original very high value. Records 2 a and 2 b show what happened when the solution in the tube was replaced by potassium-free Ringer's solution. Both deflexions were increased, indicating that the membrane resistance was raised. But no quantitative conclusion could be drawn until the results were analysed numerically. The change in

membrane resistances occurred within a few seconds, since the ions only had to diffuse across about 3μ of loose connective tissue in order to reach the surface membrane. As a rule, measurements were made within 20–60 sec. from the time when the solution was changed. It was important to make the measurements quickly, since this reduced errors from slow drifts in membrane resistance or from diffusion along the nerve fibre into regions adjoining the tube. Normal Ringer's solution was replaced in the tube as soon as the measurements were complete. Records 3 *a* and 3 *b* show that the effect of potassium-free Ringer's solution was almost completely reversible.

The effect of Ringer's solution containing five times the normal amount of potassium chloride is shown by records 4 *a* and *b*. In this case there was a substantial decrease in membrane resistance, but the membrane still recovered to a level not much lower than that existing previously (5 *a* and *b*). In calculating the change in membrane resistance, the normal resistance was taken as the mean of the values before and after application of the test solution. A test was always made at the end of an experiment to ensure that the axon was still excitable. It was found that the strength of potassium chloride could not safely be increased beyond about 70 mM. without making some of the axons irreversibly inexcitable. No certain information was obtained about the strength of potassium chloride at which the axons became reversibly inexcitable, but the majority were probably excitable at a concentration of 30 mM.

Pl. 1 shows that the membrane time constant was lengthened by a solution free from potassium and shortened by one rich in potassium. This result was always obtained and indicates that the membrane capacity was relatively little affected by changes in potassium concentration. A quantitative basis for this conclusion will be provided later.

The effects shown in Pl. 1 were observed on a large number of occasions, but a numerical analysis was only performed on the four most complete experiments. The quantitative results obtained in these four experiments are shown by Text-fig. 3 in which relative membrane conductance (β^2) is plotted against the potassium concentration of the external fluid. There is considerable scatter in the experimental points, but there can be no doubt that the membrane conductance increases markedly with potassium concentration and decreases when potassium is removed. A rough estimate is that the membrane conductance is trebled when the potassium concentration is trebled and halved when all the potassium is removed from the Ringer's solution.

The measurements were not extended to strong potassium solutions for two reasons. In the first place, the membrane resistance became too small for accurate measurement and, in the second, because the results were not completely reversible. But I gained the impression that the conductance continued to increase along the curve shown in Text-fig. 3 and that any ceiling or upper limit to the conductance was far beyond the range of my measurements.
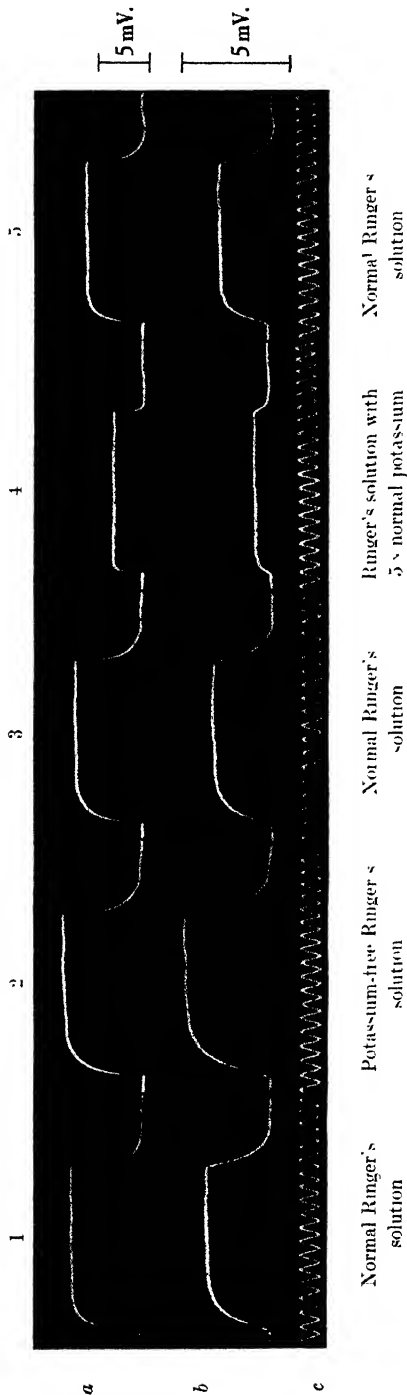
(a) Voltage recorded between points I and D, Text-fig. 1 (V_a). (b) Voltage recorded between points B and C, Text-fig. 1 (V_b). (c) 500 cyc./sec. time calibration. (1), (3) and (5) with tube containing normal Ringer (11.3 mM-K). (2) with potassium-free Ringer's solution. (4) with Ringer's solution containing 56.5 mM-K.

The effect of increasing the sodium chloride concentration in potassium-free Ringer's solution is also given in Text-fig. 3. The results show that an increase of 113 mM. has some effect on membrane conductance, but that the rise is less than that produced by addition of 11·3 mM-K$^+$ to the same solution. The relative magnitude of the effects produced by the two salts was examined in a separate series of experiments which showed that the conductance of an axon in potassium-free Ringer's solution plus 226 mM-Na$^+$ was half-way between
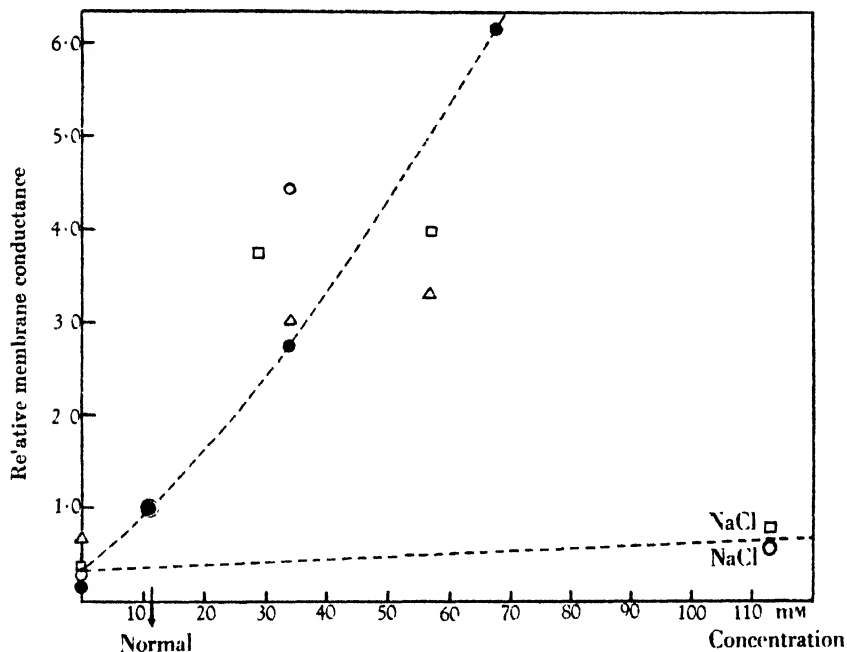
Text-fig. 3. Ordinate: relative membrane conductance (i.e. membrane conductance – normal membrane conductance). Abscissa: number of millimoles of potassium chloride (or sodium chloride) added to 1 l. potassium-free Ringer's solution. All observations were made with potassium chloride except for the two marked sodium chloride. The data plotted were obtained from four experiments each of which is denoted by a common symbol.

that existing in normal and potassium-free Ringer's solution. Thus the effect of sodium chloride on membrane conductance was roughly one-fortieth of that of potassium chloride. This result seems reasonable in view of the fact that the conductance was approximately doubled by changing from potassium-free Ringer's solution containing 493 mM-NaCl to normal Ringer's solution containing 493 mM-NaCl and 11 mM-KCl. A loose way of interpreting these results would be to say that movement of potassium ions accounts for half the membrane conductance and that the remaining part is due either to chloride or sodium. In this case the conductance would be halved if the potassium conductance were abolished by removal of external potassium. The same

argument indicates that the conductance would increase by 46 % when the sodium chloride concentration of potassium-free Ringer's solution was increased from 493 to 719. Both predictions agree approximately with the experimental findings. This hypothesis cannot be made quantitative until more is known about ionic mobilities and densities in the surface membrane. Tentative calculations suggest that the effect of potassium chloride cannot be solely due to its action in increasing the concentration of potassium in the surface membrane, and that some more definite type of permeability change must be also involved. But the interest of the results does not rest only with their quanti-
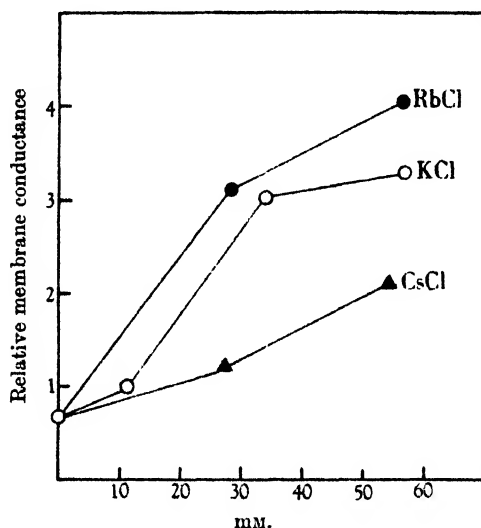
Text-fig. 4. Relation between membrane conductance and concentration of rubidium chloride, caesium chloride and potassium chloride in Ringer's solution. The abscissa gives the number of millimoles added to 1 l. of potassium-free Ringer's solution.

tative interpretation. From a physical point of view it is remarkable that potassium and sodium should have such widely different actions on the relatively simple property of membrane conductance. Another surprising but familiar aspect of the potassium effect is that the membrane conductance should be altered so drastically by small change in the external potassium concentration when the inside of the membrane is normally in contact with a solution containing at least 100 mM-K. The effect of small concentrations of external potassium on the membrane conductance will be used in a later paper as a biological method of detecting potassium leakage during activity.

Text-fig. 4 shows the relative effects of rubidium, potassium and caesium chlorides. The results indicate that these salts produce approximately equal effects when they are added in the ratios 0·8:1·0:2·2. The relatively large effect of rubidium chloride was surprising, since the action of cations in biological

systems is usually given in the order K > Rb > Cs (see Höber, 1945; Cowan, 1934). But Wilbrandt (1937) stated that rubidium is more effective than potassium in depressing the resting potential of *Maia* nerve, and Collander (1937) showed that, out of seventeen species of plant tested, fourteen concentrated rubidium to a greater extent than potassium. Quantitative results of the kind shown in Text-fig. 4 were only obtained on one occasion, but the relative effects of these three salts were confirmed by a number of qualitative observations.

The effect of lithium chloride was found to be very similar to that of sodium chloride. Thus it appears that equal increments of membrane conductance are produced by addition of salts in the following approximate molar ratios: RbCl, 0·8; KCl, 1·0; CsCl, 2·2; NaCl, 40; LiCl, 40.

The effect of acetylcholine choride was investigated in a few preliminary experiments. No appreciable increment in conductance was produced by solutions in concentrations up to 1 % (55 mM.). The acetylcholine solutions were made in unbuffered sea water. A test on the rectus abdominus muscle of the frog (for which I am indebted to Mr Comline) showed that no appreciable hydrolysis took place during the period of the experiment.

Part II

Changes in the membrane conductance of an axon immersed in oil can be measured with the help of one of the elementary equations of cable theory (see Hodgkin & Rushton, 1946. equation (6·1)):

$$\text{Membrane conductance per unit length} = \left(\frac{I}{V_A}\right)^2 \frac{1}{4}\cdot\frac{r_1^4}{(r_1+r_2)^3}. \tag{17}$$

Here V_A is the voltage recorded at the polarizing electrode minus the small potential difference resulting from current flow through the electrode resistance; the remaining symbols have their usual significance.

In principle, the change in membrane conductance can be determined from the change in V_A produced by application of a test solution. But there are two objections to this procedure. The first is that a complicated correction for the effect of the solution on r_1 must sometimes be made. The second is that the composition of the external fluid can only be changed by dipping the axon into the test solution. This disturbs the axon mechanically and may alter the position of the polarizing electrode. Frequent control 'dips' into a normal solution must be made, and the experiment should be abandoned if these do not give satisfactory results. An important factor was found to be the state of the interface between the oil and sea water. The surface should be free from dust and from the surface-active proteins which leak out of the nerve trunk during dissection. The results obtained by the dipping technique were so interesting that the method was used extensively in spite of all its difficulties.

A convenient arrangement is shown in Text-fig. 5. The axon was looped over electrodes C and D and under electrode A, so forming a V. It was dipped in the test solution to the level shown by lowering the electrode assembly with a screw control; after about 20 sec. it was raised into oil as gently as possible. The axon was observed with a binocular microscope during this operation in order to ensure that it passed through the interface cleanly and that drops of
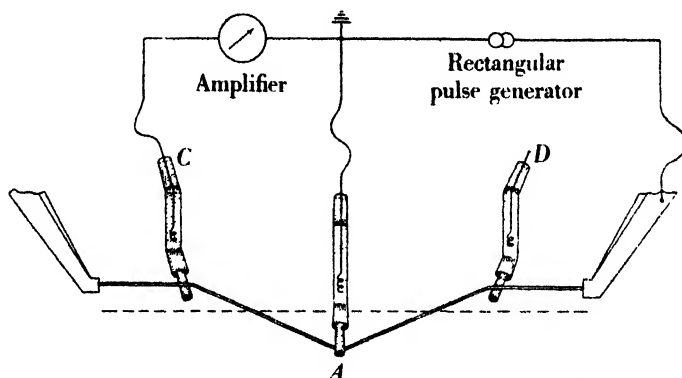
Text-fig. 5. Diagram of apparatus used for measuring effect of 'dipping' axons on membrane conductance. See text for letter references.

fluid were not picked up by the electrode. The mechanical strain imposed by an arrangement of this kind did not seem to have any serious effect on the viability of an axon, since nerve fibres in the position shown have been observed to conduct impulses for 24 hr.

Text-fig. 6 illustrates the result of an experiment designed to test the action of isotonic sea water containing 30 mM-KCl. The specific resistance of this solution differed from sea water by less than 1 %, so that the term $r_1^4/4 \ (r_1 + r_2)^3$ could be taken as constant. The control dip into sea water produced a slight apparent increase in membrane conductance which might have been due either to a genuine membrane change or to an alteration in the recording conditions. The dip into 30 mM-KCl increased the membrane conductance to 3·3 times its original value, but this increase was not maintained, and the conductance returned smoothly towards its original value. After 10 min. the axon was dipped into sea water, and this process completed the return to the original conductance. The smooth recovery of conductance was not observed in the experiments using a large volume of solution: in that case the conductance remained approximately constant or increased slowly. The recovery in oil can be most easily explained by assuming that the minute quantity of excess potassium present in the external fluid is absorbed by the axis cylinder. Potassium could be absorbed at the same rate in a large volume, but this would change neither the internal nor the external concentrations appreciably, since

the total quantities of internal and external potassium are very large compared to the amount absorbed in a few minutes. This is a reasonable hypothesis, since Fenn (1936) and Boyle & Conway (1941) have shown chemically that excess potassium is absorbed by muscle fibres. The relatively rapid rate of recovery is also to be expected, since the volume of external fluid is only about one-third of that of the axis cylinder, and the concentration of potassium outside is low
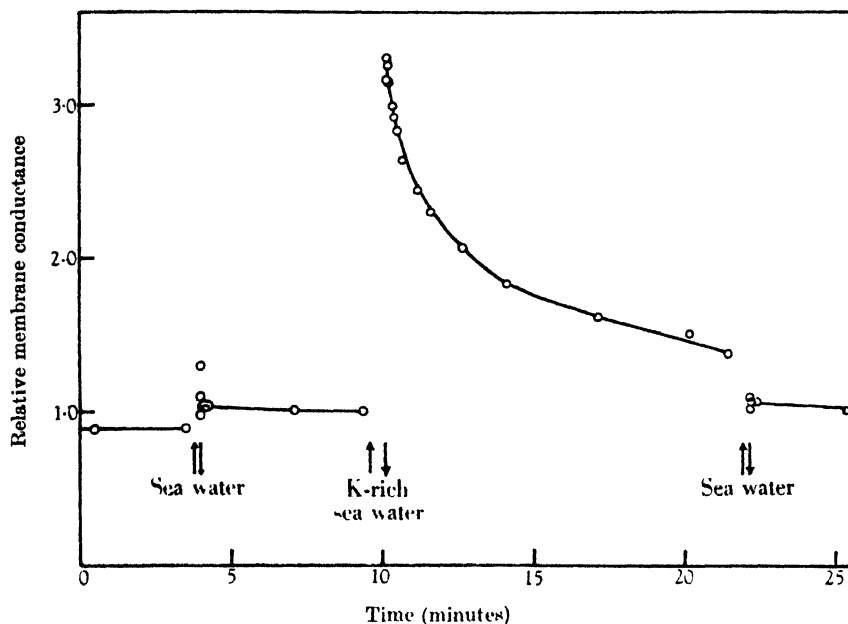
Text-fig. 6. Effect of dipping axon into sea water and into an isotonic sea-water solution containing 30 mM-K.

compared to that inside the fibre. A small absolute rate of potassium transfer will therefore clear the external fluid of potassium excess in a relatively short time. The absorption of excess potassium became very slow when the apparent concentration was still well above the normal value, and it was usually only possible to restore the membrane conductance to its original value by dipping the axon into sea water. A result of this kind might be expected on theoretical grounds, but there was reason to believe that the lack of complete recovery may have been due to an experimental factor. When the polarizing electrode was raised from a potassium-rich solution into oil it carried with it a film containing excess potassium. This excess potassium would not have been easily absorbed, since the volume of the electrode was large compared to the volume of axis cylinder in contact with the electrode.

Evidence for the view that electrode KCl was hindering complete recovery is provided by an experiment in which the dipping technique was performed in a different way. The axon was arranged to run horizontally over all the

electrodes and a record of V_A obtained in a normal condition. The polarizing electrode was then removed and the axon stroked with a drop of potassium-rich solution which hung from a glass capillary tube controlled by a micro-manipulator. The polarizing electrode was replaced and the new value of V_A determined as quickly as possible. This operation required considerable dexterity and was only used occasionally. Text-fig. 7 illustrates the results obtained with *Carcinus* Ringer's solution containing 34 mM-K. The relative
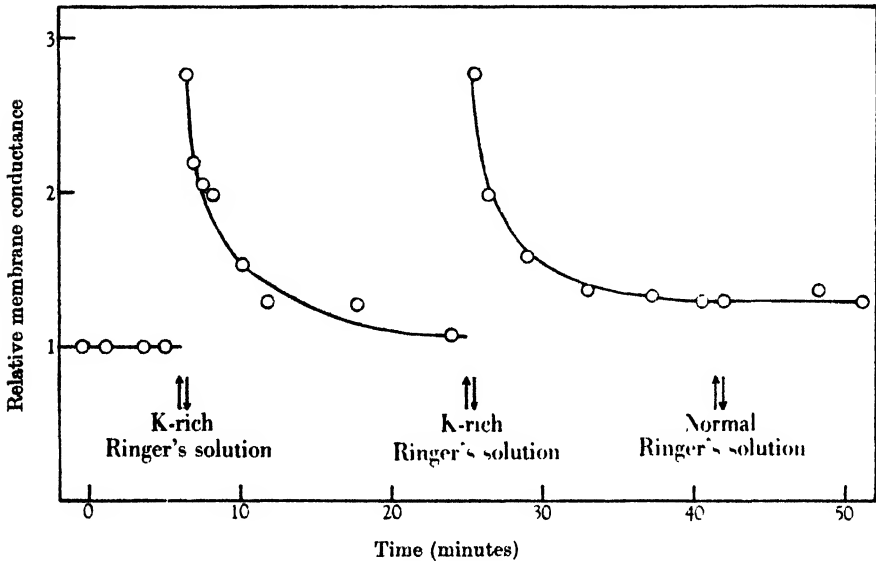
Text-fig. 7. Effect of stroking axon with potassium-rich Ringer's solution containing 34 mM-K and with normal Ringer's solution.

membrane conductance rose to 2·8 and then returned to a steady level which was only slightly greater than that existing previously. In this experiment the electrode picked up no test fluid, so that recovery would be helped rather than hindered by diffusion into or out of the electrode. When application of the test solution was repeated, the conductance again rose to 2·8 and again returned nearly to its original level. Application of normal Ringer's solution at the end of the experiment produced no further change in conductance.

The only simple way of explaining the results shown in Text-figs. 6 and 7 is to assume that the excess potassium disappeared from the layer of fluid surrounding the axon. This potassium must have moved either into the axis cylinder or into the electrode. The second process would be too slow to account for more than a fraction of the recovery observed and does not explain the recovery shown in Text-fig. 6. There can be little doubt, therefore, that the recovery of membrane conductance reflects the absorption of excess potassium by the nerve fibre.

The initial effect of potassium on membrane conductance was similar to that observed in the tube experiment. The results of a series of experiments with sea water containing 30 mM-KCl are shown in Table 1; they are plainly consistent with those given in Text-fig. 3. An attempt was made to measure the absolute rate of potassium absorption by the axon. A curve of the type shown in Text-fig. 6 gives the rate at which the potassium-ion concentration is

TABLE 1. Ratio of membrane conductance in potassium-rich solution to normal membrane conductance (β^2)

The figures give the relative membrane conductance immediately after dipping into isotonic sea water containing 30 mM-K⁺. Brackets indicate that measurements were made on the same axon. Results obtained with the stroking technique are not included because too long an interval elapsed between removal of the potassium-rich drop and the first electrical measurement.

3·4	2·9	
3·3 ⎤	2·1	Average 2·8
3·3 ⎬	2·7 ⎤	
3·0 ⎦	2·0 ⎦	

diminishing in the external fluid and the absolute rate can be calculated if the axon diameter and the volume of external fluid are known. The axon diameter was measured microscopically and the external volume estimated from the resistance of the external fluid. The layer between the axis cylinder and the oil in which it was immersed consisted of loose connective tissue and sea water. The space available for external potassium may reasonably be taken as the

TABLE 2

Apparent membrane conductance in sea water containing 30 mM-K (micromhos cm.⁻²)	Apparent rate of absorption of K in mol.cm.⁻² sec.⁻¹ from solution containing 30 mM-K
366	$3·6 \times 10^{-11}$
⎧ 808	$12·7 \times 10^{-11}$
⎩ 1002	$7·8 \times 10^{-11}$
⎧ 571	$29·5 \times 10^{-11}$
⎩ 1505	$5·2 \times 10^{-11}$
Average 850	$11·8 \times 10^{-11}$

Bracketed figures obtained on same axon.

quotient of the resistance per unit length and the specific resistivity of sea water. The external resistance per unit length was measured by the method described in the preceding paper (Hodgkin, 1947), and an average volume of about 3×10^{-6} c.c. was obtained. This volume corresponds to a layer of sea water about 3μ in thickness and agrees reasonably with the results of direct optical measurement of the thickness of the connective tissue layer. Individual measurements of external volume and axon diameter were made in each experiment. The absolute rates at which potassium was absorbed when the concentration was increased from 9·8 to 30 mM. are given in Table 2. The individual determinations must have been subject to large random errors, but

the results give an order of magnitude of 10^{-10} mol. cm.$^{-2}$ sec.$^{-1}$, for the rate of absorption of excess potassium. An estimate is also given of the membrane conductance which corresponded to this rate of absorption. These values were obtained from the normal membrane conductance and the increase in conductance given by each potassium curve.
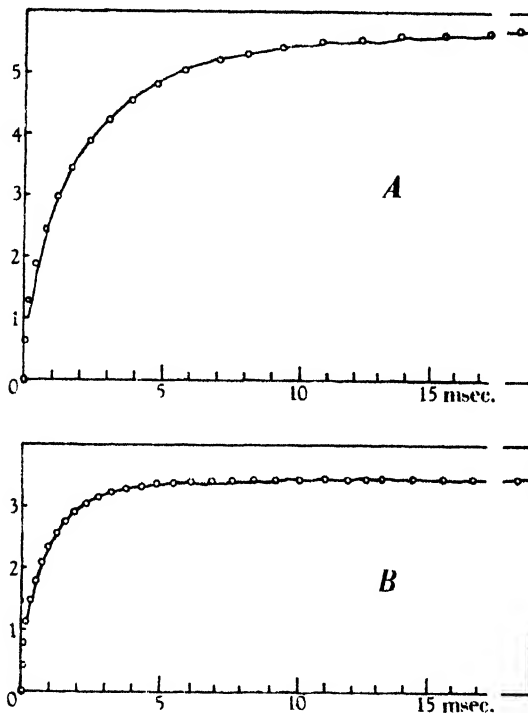
Text-fig. 8. Time course of potential change at polarizing electrode when current is suddenly applied. A is the record obtained from a normal axon and B that obtained immediately after stroking the axon with Ringer's solution containing 34 mm-K. The continuous lines are tracings of the experimental records and the circles are theoretical points calculated from equation 18 with $\tau_m = 4\cdot78$ msec. in A and $\tau_m = 1\cdot90$ msec. in B. A very rapid shock artifact at the beginning of each record has been omitted. The potential drop across the polarizing electrode (which amounted to $0\cdot17$ unit) has been subtracted from both records. Two oscillograph traces are superposed in A.

An axon immersed in potassium-free Ringer's solution might be expected to leak potassium until the concentration of potassium in the Ringer's solution returned to normal. Some indication of this effect was observed in preliminary experiments, but the results were marred by the unsatisfactory nature of the control 'dips' and are not presented here.

Experiments with the tube electrode showed that the potassium-rich solutions altered the membrane capacity less than the membrane resistance.

This result could not be made quantitative because the complicated boundary conditions introduced by the tube only allow manageable equations to be obtained when a steady state is considered. An attempt to discover whether potassium had any effect on membrane capacity was made with the 'stroking' technique which has just been described. Since a theoretical equation is only available for an infinitesimal electrode, care was taken to reduce the electrode region to about 100μ. Text-fig. 8A shows the potential time curve obtained when current is suddenly applied. The circles are theoretical points calculated by the equation for an infinitesimal electrode (Hodgkin & Rushton, 1946), viz.

$$(V_A)_t = (V_A)_{t=\infty}\mathrm{erf}\,[\sqrt{(t/\tau_m)}]. \tag{18}$$

τ_m is the membrane time constant and is equal to the product of membrane resistance and capacity. Here τ_m was taken as 4·78 msec.

The polarizing electrode was removed and the axon stroked with *Carcinus* Ringer's solution containing 34 mM-K. The record obtained when the electrode was replaced is given in Text-fig. 8B. In this case the theoretical equation was plotted with a time constant of 1·90 msec. The ratio of the absolute heights of $(V_A)_{t=\infty}$ indicates that the membrane conductance increased in the ratio 2·74:1 when equation (18) is applied on the assumption that the term $r_1^4/4\,(r_1+r_2)^3$ remained constant. A rather uncertain correction for the change in r_1 indicates that the membrane conductance change was 2·40. These figures show that the capacities for normal and treated axons were in the ratios 1:1·09 or 1:0·95; the second ratio being that obtained after correction for a change in r_1. A similar experiment gave the following figures:

> Membrane conductance ratio 2·46 or 2·15,
> Membrane capacity ratio   1·18 or 1·04.

The accuracy of the method does not allow any certain conclusion to be drawn, but the capacity clearly changes much less than the conductance and may well be unaffected by potassium.

DISCUSSION

Potassium absorption and the ionic steady state. The absorption of excess potassium may be thought of as an active process of a secretory type. But it can equally well be explained by the type of theory proposed by Boyle & Conway (1941). Nerve and muscle appear to contain a large quantity of unidentified organic anions. These establish a Donnan membrane equilibrium because they cannot diffuse through the surface membrane. Sodium ions either cannot pass through the membrane or are actively extruded by a secretory process (cf. Krogh, 1946). A high concentration of potassium is therefore needed to balance the indiffusible anions. It makes little difference to the potassium

distribution whether sodium is kept out by an active process or by a semi-permeable membrane. In either case the ratio of the activity of potassium inside the membrane to that outside should be given by the expression $e^{EF/RT}$, where E is the resting potential difference across the membrane and the other symbols have their usual significance. The activity ratio could only exceed the value defined by the resting potential if active transfer of potassium took place. At present there is no clear evidence to show that the activity ratio does exceed this ratio, so that neglect of active transfer seems reasonable in a working hypothesis. The absorption of excess potassium can be readily explained on this basis. Potassium would be absorbed as potassium chloride if the view that the membrane is permeable to K^+ and Cl^- but impermeable to Na^+ is accepted. Addition of a potassium-rich solution raises the $[K] \times [Cl]$ product in the external fluid and absorption would continue until this product were reduced to the internal $[K] \times [Cl]$ value. Boyle & Conway (1941) suggested that their theory did not apply to peripheral nerve, because it could not explain the high chloride concentrations reported by Bear & Schmitt (1939) and Webb & Young (1940) for squid axoplasm. However, Steinbach (1940, 1941) describes experiments which indicate that the chloride concentration in freshly dissected axons is only about one-quarter of that observed by Bear & Schmitt. He also reports evidence which suggests that the squid axon is permeable to chloride. Steinbach himself does not appear to subscribe to Conway's theory, but his results agree much more closely with the predictions of the theory than any reported previously.

Sodium must be actively extruded at a high rate if the nerve fibre is considered to be permeable to sodium ions. In this case excess potassium would, in part, be absorbed in exchange for the sodium extruded. A more general way of looking at the situation is to say that the resting potential is maintained by an unknown process and that excess potassium is sucked through the membrane because the potential gradient exceeds the concentration gradient. The identity of the ion which accompanies or exchanges with potassium cannot be specified until the resting potential mechanism is more closely defined.

The absolute rate of potassium absorption should be related to the membrane conductance if potassium is absorbed as ionized potassium chloride. The theoretical rate cannot be calculated with certainty unless simplifying assumptions about the structure of the nerve membrane are made. Nor can any close agreement between theory and practice be expected, since the measurements of absolute rate and membrane conductance were subject to large experimental errors. But the comparison is worth making, since a disagreement in the order of magnitude might disprove the idea that potassium and chloride are absorbed in ionized form. The simplest method of estimating the rate of potassium absorption is to assume that the mobility of K^+ is equal to that of Cl^- and that Planck's (1890 a, b) assumptions for liquid junction potentials may

be applied to the nerve membrane. In this case the rate of potassium absorption should be given by the following expression:

$$y = \frac{G_4 RT}{4F^2} \log_e h, \tag{19}$$

where y is the rate of potassium absorption in mol.cm.$^{-2}$ sec.$^{-1}$, h is the ratio of the external potassium concentration to that which is normally in equilibrium with the axon, G_4 is the membrane conductance per sq.cm, and R, T and F have their usual significance. When the external potassium concentration is raised from its normal value of 9·8 mM. to 30 mM., equation (19) becomes

$$y = \frac{G_4}{F} \times 6 \cdot 92 \ mV. \tag{20}$$

A more general theory which did not impose the restriction of microscopic electroneutrality gave a rate which was also proportional to membrane conductance, but was about 25 % lower than that predicted by equation (20). The average membrane conductance of the axons considered in Table 2 was 850 micromhos cm.$^{-2}$ when the external concentration of potassium was 30 mM. A rate of $6 \cdot 1 \times 10^{-11}$ mol.cm.$^{-2}$sec.$^{-1}$ is predicted when this value is substituted in equation (20). The average rate actually found was $11 \cdot 7 \times 10^{-11}$ mol.cm.$^{-2}$sec.$^{-1}$ The agreement in order of magnitude is interesting, but cannot be regarded as positive evidence for the view that potassium is absorbed entirely as ionized potassium chloride. Nor can the fact that there appeared to be little correlation between the individual values of conductance and absorption rate be taken as evidence against such a hypothesis, since the variation shown by Table 2 may have been largely due to experimental error.

The most serious obstacle to theories of the ionic steady state is to decide not how potassium is absorbed against a concentration gradient, but how sodium is extruded and the resting potential maintained. The last two problems are likely to be related, since an active extrusion of sodium would tend to make the inside of the axon negative and so maintain or augment the resting potential. At present there are two views about the importance of the sodium extrusion process which must almost certainly be at work in many living cells. According to Conway (1946) the surface of a muscle fibre is virtually impermeable to sodium ions, so that the active excretory process operates very slowly and may be neglected in short-term experiments. According to Krogh (1946) many cells including muscle are much more permeable to sodium than to potassium, so that sodium must be actively extruded at a very high rate. The experiments described in this paper tend to favour Conway rather than Krogh's view. It would be hard to imagine why addition of potassium chloride to sea water increased the membrane conductance 40 times more than the addition of an equivalent quantity of sodium chloride, if Na$^+$ could penetrate more easily

than K⁺. Similar conclusions can be drawn from the effects of K^+ and Na^+ on the resting potential (Osterhout, 1931, Höber, 1945). Electrical evidence of this kind suggests that potassium ions have a greater mobility or solubility in the surface membrane than sodium ions, but they do not give any information about the permeability of the membrane to unionized compounds, and it is conceivable that sodium may be transported in unionized form. An assumption of this kind might help to resolve the apparent discrepancy between the results of Conway's experiments and the facts cited by Krogh (Conway, 1946; Krogh, 1946). The same assumption could also be used to explain why sodium is normally kept at a low concentration in the interior of a nerve or muscle fibre. It is possible to imagine that the small amounts of sodium which penetrate the cell in ionic form are slowly extruded in unionized form by combination with a lipoid-soluble carrier.* Such a state of affairs could arise if there were a continuous production and leakage of the sodium carrier or if the carrier remained in the cell membrane, but was altered chemically in a cyclical manner as a result of metabolic activity. According to such a view potassium would be likely to predominate over sodium in the cell interior for two reasons. The first is that the tendency of potassium ions to enter should be greater than that of the more heavily hydrated sodium ions; and the second, that the tendency of sodium to combine with a lipoid-soluble carrier might conceivably be greater than that of potassium, since cations are said to give rise to covalent linkages more readily when their atomic number is small (Sidgwick, 1927). A cell operating in this way would appear to be impermeable to sodium when tested by Conway's methods (Conway, 1946; Conway & Moore, 1945), since any sodium which entered would be actively extruded. On the other hand, it would appear to be permeable to radioactive sodium, since there would be a continuous flux of sodium across the surface membrane. Further, it can be shown that application of the simple permeability equation used by Krogh (1946) leads to results which are completely misleading. If, as Krogh maintains, there is an active secretory process at work in living cells, then this process cannot be ignored in calculations which purport to show that cell membranes are more permeable to sodium than they are to potassium.

Potassium ions and excitation. A decrease in the electrical threshold might be expected to follow the application of potassium-rich solutions, since potassium ions depolarize the nerve membrane. An action of this kind has been reported by several workers, but the converse effect has also been observed frequently (for references see Fenn, 1940). There must always be an increase in threshold at some stage, since conduction is eventually blocked by high

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* The sodium carrier might take the form of a long molecule or micelle in the surface layer as suggested by Lundegårdh (1940). From many points of view the hypothesis outlined here does not differ materially from that discussed by Krogh (1946), to whose article the writer wishes to express his indebtedness.

potassium-ion concentrations. In my experience an increase in threshold almost invariably follows the application of potassium-rich solutions to *Carcinus* axons. It is true that local applications of concentrated potassium solutions sometimes produce injury discharges, but in such cases impulses arise from the regions adjacent to that treated with potassium-rich solution as a result of local-circuit action, and it cannot be said that the nerve membrane itself is more excitable as a result of increased potassium. The depressant action of potassium is partly explained by its effect on membrane resistance. When a weak current is applied to a nerve fibre the depolarization produced at the cathode is proportional to the square root of the membrane resistance. Anything which lowers membrane resistance must increase the threshold current, unless it has a specific effect on the critical level of depolarization required for excitation. Some of the depressant action of potassium can be attributed to its effect on membrane resistance, but preliminary experiments suggest that this cannot be the complete explanation. Electrical measurements show that potassium increases not only the current required to produce a given depolarization, but also the level of depolarization at which a local response becomes unstable and turns into a spike. An explanation of this effect cannot be given until more is known about the nature of the process responsible for generating local and propagated responses.

The experimental results described in this paper may be expected to hold for other non-medullated fibres and possibly for muscle fibres, but it is doubtful if they apply to medullated nerve. Danielli's (1939) work suggests that the resistance of the myelin sheath is not appreciably altered by changes in potassium concentration, and it may be that the physiological effects of potassium arise at the nodes of Ranvier.

SUMMARY

1. A method of observing the effect of ions on the conductance of the surface membrane of a nerve fibre is described. The effect of potassium and related ions on membrane conductance was investigated with isolated axons from *Carcinus maenas*.

2. Small changes in external potassium concentration caused large and rapidly reversible changes in membrane conductance. The conductance was increased roughly threefold when the potassium concentration in *Carcinus* Ringer's solution or sea water was trebled and was reduced to about one-half by removal of potassium.

3. Similar increments of conductance were produced by adding salts in the following molar ratios: RbCl, 0·8; KCl, 1·0; CsCl, 2·2; NaCl, 40; LiCl, 40.

4. Changes in external potassium chloride concentration had relatively little effect on the membrane capacity.

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5. The increase in conductance produced by excess potassium chloride was not maintained if the volume of external fluid was made small by immersing the axon in oil. The recovery in conductance was almost certainly due to absorption of excess potassium by the axon against a concentration gradient. The average rate of absorption of potassium was approximately 10^{-10} mol./sec./sq.cm. membrane when the external potassium was increased from its normal value of 9·8 to 30 mM.

I wish to express my indebtedness to Mr A. F. Huxley for his assistance in some of the experiments and calculations. Many of the ideas presented in the discussion arose in conversation with him.

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POTASSIUM LEAKAGE FROM AN ACTIVE NERVE FIBRE

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The inorganic composition of animal tissues has been the subject of investigation since the time of Berzelius (1807) and Von Liebig (1847). There is now general agreement that the concentration of potassium is much higher in the interior of a nerve or muscle fibre than it is in plasma, whereas the concentrations of sodium and chloride are considerably less than those in plasma. Some of the clearest evidence is provided by studies of the axoplasm of the giant axon of the squid. Here the potassium concentration in the axoplasm of a fresh axon may be at least twenty times that in squid blood, whereas the sodium and chloride may be present in less than one-tenth of their concentrations in blood (Steinbach & Spiegelman, 1943; Steinbach, 1940, 1941). Figures closer to those in blood have been observed by other workers (Webb & Young, 1940; Bear & Schmitt, 1939), but can be explained by assuming that ionic exchange occurs during the process of isolating the axon. The differences in ionic concentration are almost certainly related to the difference in electrical potential between the inside and outside of the axon. The way in which the axon maintains the ionic concentration difference and the resting potential is unknown, but the difference in ionic concentration clearly provides a source of energy which might be used to transmit nervous impulses. This idea was an essential part of Bernstein's (1912) hypothesis and has been widely used by physiologists. Bernstein assumed that the resting membrane was permeable to potassium but not to other ions, so that the concentration difference would make the inside of the fibre negative with respect to the outside. During activity the membrane was assumed to become permeable to other ions with the result that it was depolarized and gave a negative action potential. The work of Boyle & Conway (1941) indicates that resting muscle fibres are permeable to chloride as well as to potassium, and a similar conclusion can be drawn from the studies of Shanes (1946) and Steinbach (1941) on non-medullated nerve. This result does not seriously affect Bernstein's theory, since the concentration difference of chloride is in the opposite direction to that of potassium. But it suggests that exchange of potassium and sodium is a more likely source of energy for nervous transmission than the leakage

of potassium chloride. The idea that the nerve membrane became more permeable during activity was greatly strengthened by the demonstration of a transient decrease of membrane resistance during activity (Cole & Curtis, 1939). On the other hand, the work of Curtis & Cole (1942) and Hodgkin & Huxley (1939, 1945) indicated that the action potential was not a simple depolarization, since the membrane potential was found to reverse during activity. This result suggests that the mechanism of activity is more complicated than was formerly thought, but it does not preclude the possibility that the energy required for transmitting an action potential is at least partly derived from potassium leakage. Observations and measurement of potassium leakage during activity are therefore of great importance for theories of nervous transmission. Direct chemical estimations of potassium leakage are beset with difficulties, but have been used with success by several workers. Cowan (1934) showed that *Maia* nerve lost a significant quantity of potassium to sea water when it was stimulated to fatigue. Young (1938) observed a similar leakage in the leg nerve of *Limulus*. Arnett & Wilde (1941) showed that stimulation of medullated nerve for 60 min. at 3000 impulses/min. caused an appreciable potassium leakage, but they failed to find any significant effect after 30 min. of stimulation at the same frequency. Loss of potassium from active muscles has been reported by several workers but others have obtained negative results (see Fenn, 1936, 1940). The general impression given by the literature is that prolonged stimulation may often cause leakage of potassium. But there is no evidence as to the magnitude or the time course of the leakage; nor is there any certainty that potassium leakage is a normal and invariable accompaniment of activity. The present paper is concerned with the results obtained by a biological method of estimating potassium leakage. The method is indirect, but has the advantage that it is capable of giving a continuous record of the potassium concentration in the external fluid.

An isolated axon from *Carcinus maenas* is surrounded by a layer of saline which is only a few micra in thickness when it is immersed in oil. Electrical and optical estimates indicate that the volume of external fluid which surrounds 1 cm. length of axon is about 3×10^{-6} c.c. The potassium concentration in this layer is normally the same as that in sea water (9·8 mM.), so that the total amount of external potassium is about 3×10^{-11} moles/cm. In the preceding paper it was shown that the conductance of the nerve membrane was very sensitive to the external concentration of potassium (Hodgkin, 1947b). A rough estimate was that the membrane conductance was trebled when the external potassium concentration was increased from 9·8 to 30 mM. The membrane conductance should therefore change markedly when a train of impulses passes down the axon, if there is any appreciable leakage of potassium. Changes in membrane conductance might arise from other causes besides leakage of potassium, but this possibility can be controlled to some

extent by observing what happens to the membrane when it is surrounded by a large volume of fluid. Such experiments cannot eliminate the possibility that the effects observed are caused by leakage of a substance which affects the nerve membrane in a potassium-like manner. But the results should set an upper limit to the potassium leakage and are in any case of interest because they are concerned with a new experimental effect.

METHOD

Most of the equipment used was similar to that employed in previous work and will not be described here. The essential features of the electrode assembly are shown in Text-fig. 1. Rectangular pulses of current were applied to the nerve through 100 MΩ. The extrapolar potentials (electrotonus) produced by these pulses were recorded from the polarizing electrode (A) or from
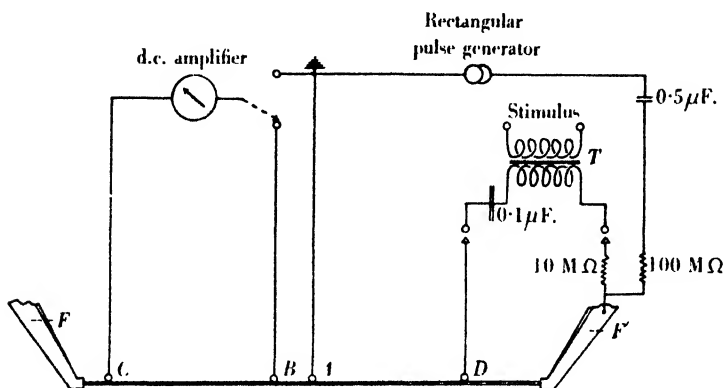
Text-fig. 1. Diagram of apparatus used for measuring the cumulative effect of activity upon membrane conductance. A, B, C, D, non-polarizable electrodes of silver chloride type; F and F', forceps for holding ends of axons.

a second electrode (B) placed at 1–2 mm. from the polarizing electrode. The nerve was stimulated through a transformer (T) which could be connected by means of a low-capacity switch placed a few centimetres from the preparation. In some of the early experiments the stimulus was switched on and off by means of a key in the primary circuit of the transformer. This method of switching introduced some error in the time course of the extrapolar potential, but did not materially affect the measurement of membrane conductance. The stimulus consisted of a train of shocks the frequency of which could be varied between 1 and 500/sec. It was generated by means of a simple thyratron circuit. The usual precautions were taken to avoid the artifacts which arise from electrical leaks or lack of balance in the amplifier. A continuous record of membrane conductance was obtained by photographing successive oscillograph frames on a 35 mm. film which was moved at a rate of about 2 cm./sec.

The experiments to be described in this paper required that axons should be capable of transmitting a large number of impulses without changing their properties unduly. Care was taken to use only those axons which were in good physiological condition. 'Good' axons are free from kinks and constrictions, their axoplasm is perfectly clear, and they often show a marked corrugation at the surface of the axoplasm. Physiological characteristics of such axons are large action potential, low threshold, high membrane resistance and ability to give prolonged repetitive discharges when constant currents are applied. The state of the experimental animals was an important factor in obtaining axons of good viability. The aquarium was replenished at frequent intervals with crabs obtained freshly from the sea and sluggish animals were rejected.

Preliminary experiments

The cumulative effect of stimulation on membrane conductance is shown by Pl. 1. Record A 1 gives the potential produced by application of a rectangular pulse of current with a duration of 42 msec. The potential does not rise to its maximum instantaneously because the nerve membrane takes an appreciable time to charge. In this case the membrane time constant was about 6 msec. The amplitude of the steady deflexion produced by the current increases with membrane resistance but is not directly proportional to it. A qualitative indication of the effect of activity on membrane resistance is obtained by observing whether the amplitude of the deflexion in A 1 can be changed by stimulation. The effect of stimulation is seen in A 2. Soon after record A 1 had been obtained a train of impulses was initiated by connecting the terminals of the stimulating transformer to the nerve. The stimulus was left on for 30 sec., and photographic recording showed that approximately 3500 impulses travelled throughout the entire length of the nerve fibre. During the tetanus the membrane resistance could not be measured because of the confusion produced by the action potentials and the transient decreases in electrical resistance which accompanied them. But records taken immediately after activity showed that the resistance had been decreased, as may be seen from the small size of the deflexion in A 2. During the ensuing period the resistance returned gradually to its former level until, at 13 min. after activity, it was almost completely restored to its original level. These changes could have been produced by a leakage of potassium during the tetanus followed by a reabsorption during the subsequent period of recovery. But several other explanations of the effect are possible, and the interpretation in terms of potassium leakage was not pursued until a number of preliminary experiments and controls had been performed.

The second row of records in Pl. 1 were obtained under conditions which were identical with those just described, except that the distance between polarizing and recording electrodes was increased. The equations of cable theory predict that the effect of the tetanus on the extrapolar potential should increase as the recording electrode is moved away from the polarizing electrode; the records in Pl. 1 show that this prediction is verified. The magnitude of the change in membrane conductance can be calculated, if it is assumed that the resistances of axoplasm and external fluid are not altered by stimulation. An experimental justification of this assumption will now be presented.

The steady potential recorded in the extrapolar region should obey the following relation:

$$V_B = \frac{r_1^2 I e^{-l\sqrt{[g_4(r_1+r_2)]}}}{2\,(r_1+r_2)^{\frac{3}{2}}\,g_4^{\frac{1}{2}}}, \tag{1}$$

where r_1 and r_2 are the external and internal resistances per unit length, g_4 is the membrane conductance per unit length, l is the distance between the polarizing and recording leads, and I is the applied current. The following relation can be derived from this equation, if it is assumed that activity changes only the membrane conductance:

$$\log_e \left[\frac{V_B \text{ before activity}}{V_B \text{ after activity}} \right] = \log_e \beta + \frac{(\beta - 1)\, l}{\lambda}. \tag{2}$$

Here β^2 is the ratio of the membrane conductances after and before activity, while λ is the space constant in the normal axon and is equal to $[g_4\,(r_1 + r_2)]^{-\frac{1}{2}}$.
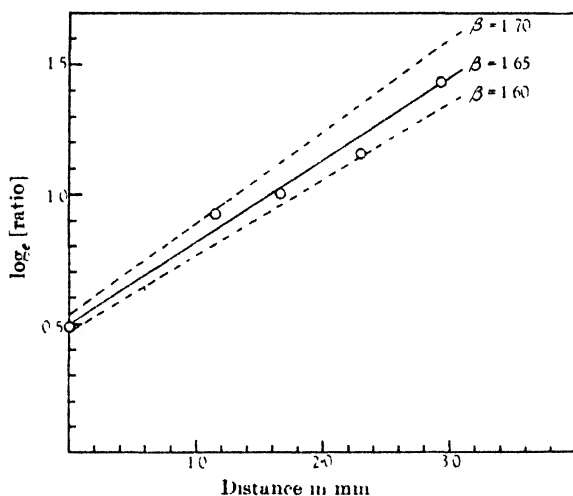
Text-fig. 2. Abscissa: distance between polarizing and recording electrodes. Ordinate: $\log_e$. (Extrapolar potential before activity/Extrapolar potential 5 sec. after activity.) The nerve was stimulated with a 30 sec. tetanus of frequency about 117/sec. The straight lines are drawn according to equation (2).

This equation was tested by measuring the change in potential produced by a tetanus at different distances from the polarizing electrode. The logarithm of the ratio of the potentials before and after activity is plotted against distance in Text-fig. 2. The value of λ in a normal nerve was obtained by measuring the spatial distribution of potential in the usual manner. Equation (2) predicts that all the experimental points should fall on a straight line whose slope is determined only by λ and the intercept of the line on the ordinate. A severe test is therefore imposed on the experimental results. Text-fig. 2 shows that the experimental points do not fall exactly on a straight line, but they depart from it only in a random manner. Thus all the points fall between the lines determined by $\beta = 1.6$ and $\beta = 1.7$. Such a result could

only have been obtained if the change in resistance had been largely confined to the membrane element. Experiments of this kind were not sufficiently accurate to exclude rigidly the possibility of there being a small change in external or internal resistance, but they make it rather unlikely. Further evidence for this view came from the fact that the parallel resistance of external fluid and axis cylinder was changed by less than 2 % by a tetanus which produced a threefold increase in membrane conductance.

The change in membrane conductance produced by a tetanus could be estimated from a set of points of the type shown in Text-fig. 2. But this procedure was too elaborate for ordinary use, since it involved a number of complicated measurements. A simple method of determining the change in membrane conductance was to record the potential difference at the polarizing electrode of an applied current. This consisted of the small p.d. resulting from the flow of current in the electrode and a p.d. which was proportional to the membrane potential under the electrode and will be called V_A. The potential drop across the electrode was estimated by the method described in a previous paper and V_A obtained by subtraction (see Hodgkin, 1947 a). The ratio of the membrane conductance at any given moment to the normal conductance is given by the expression

$$\text{Relative membrane conductance} = \beta^2 = \left[\frac{V_A \text{ in normal nerve}}{V_A \text{ after activity}}\right]^2. \qquad (3)$$

This method has the advantage of simplicity, but is inaccurate when the change in membrane conductance is small. It is also open to the objection that the voltage recorded from an electrode of finite width is not exactly equivalent to the theoretical potential difference at an infinitesimal electrode. A more satisfactory method, which was used whenever possible, was to measure the change in voltage at a distance from the polarizing electrode. A value of λ was first obtained in normal nerve and β^2 could then be determined from equation (2). This method could be made extremely sensitive, since a 10 % change in conductance leads to a 22 % change in the observed potential, when the latter is recorded at 3λ from the polarizing electrode. The corresponding change in the potential at the polarizing electrode is only 5 %.

The effect of activity on membrane capacity

The records in Pl. 1 show that the membrane time constant is reduced after the tetanus. This suggests that activity changes the membrane capacity less than the membrane conductance. The extent to which the capacity alters can be estimated by comparing the experimental records with the theoretical equations of cable theory. Such a comparison is illustrated by Text-fig. 3. The curves on the left are the experimental records of the rise in potential difference at various distances from the electrode. The circles are theoretical

points calculated according to the expression derived by Hodgkin & Rushton (1946):

$$V_1 = (V_1)_{\substack{T=\infty \\ X=0}} \cdot \tfrac{1}{2} \{e^{-X}[1 - \mathrm{erf}\,(X/2\sqrt{T} - \sqrt{T})] - e^{X}[1 - \mathrm{erf}\,(X/2\sqrt{T} + \sqrt{T})]\}, \quad (4)$$

V_1 is the external potential difference,

$X = l/\lambda$,

$T = t/\tau_m$, where τ_m is the membrane time constant and is equal to membrane capacity $C_m \div$ by membrane conductance G_4.

The value of l/λ was obtained for each curve by comparing the amplitude of the potential recorded with that observed at $l = 0$. The value of τ_m was adjusted to give the best fit between experimental and theoretical curves.

The right-hand side of the figure shows the experimental curves obtained immediately after the tetanus and drawn on a larger vertical scale than those on the left. The circles were again calculated from equation (4) with new values for λ and τ_m. As before the best value for τ_m was determined by trial and error, while the new value for λ was obtained by using equation (2). (It should be remembered that activity changes λ by the factor β^{-1}.) Each pair of curves allows the ratios of membrane capacity and conductance before and after activity to be estimated. The values used in plotting the theoretical points are given in Table 1.

The analysis leaves no doubt that the membrane capacity alters very much less than the membrane conductance, but the quantitative results obtained varied with the position of the recording electrode in a manner contrary to theory. This effect is thought to have arisen in the following way. The width of polarizing and recording electrodes was $\tfrac{1}{8}\lambda$ instead of being infinitesimal as assumed in the theory. It can be shown that the effect of a finite electrode width is to increase both the apparent time constant and the apparent change in capacity, and that these effects are greatest when the potentials are recorded from the polarizing electrode. Another source of error becomes important at large distances. The beginning of each record was confused by a sharp spike caused by capacitative coupling between polarizing and recording leads. The spike itself has been omitted from the records shown in Text-fig. 3, but it left a small 'tail' which added to the true potential. The magnitude of the shock artifact remained constant, and therefore introduced an error which grew as the interelectrode distance was increased. The effect of the artifact was to make the membrane capacity appear smaller as l/λ increased. Both the absolute capacity and the capacity change therefore decreased as the recording electrode was moved away from the polarizing electrode.

The deviations from theory prevent any rigid conclusion being drawn from Table 1, but they do not obscure the fact that the capacity changes much less than the conductance, and it may be that there is no change in capacity at all.

TABLE 1. Data obtained from Text-fig. 3. λ, τ_m are values before activity;
λ', τ'_m are values 5 sec. after activity

Distance in mm. between recording and polarizing electrodes (l)	l/λ	l/λ'	τ_m (msec.)	τ'_m (msec.)	Conductance change (β^2)	Capacity change
0·0	0·0	0·0	6·13	3·07	2·64	1·32
1·15	0·49	0·86	6·74	2·67	3·03	1·20
2·30	1·03	1·69	5·75	2·13	2·68	1·00
2·93	1·418	2·35	5·20	1·6	2·74	0·84
Average	—	—	5·96	2·37	2·77	1·09
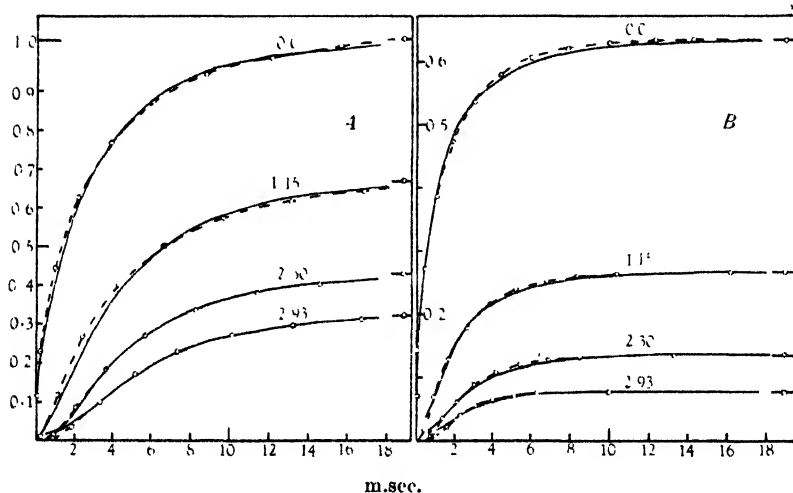
Text-fig. 3. Time course of potential change when current is suddenly applied, recorded at different distances from polarizing electrodes. *A*, before activity; *B*, 5 sec. after activity (about 3500 impulses in 30 sec.). The continuous curves are drawn from tracings of the experimental records, while the circles and dotted lines are theoretical curves drawn according to equation (4) with the values given in Table 1.

The matter might have been settled by refinements in experimental technique, but the practical difficulties were so great and the theoretical analysis so laborious that the investigation was not pursued further.

The effects of a large volume of external fluid

The experiments described in the preceding sections show that activity has a cumulative effect on the membrane conductance of an axon immersed in oil. The increase in conductance might have been caused by leakage of a potassium-like substance into the external fluid followed by a reabsorption during the ensuing period of recovery. But the effects observed might equally well have been due to a change in the axon which did not depend upon an alteration in the chemical composition of the external fluid. The relative importance of these two factors could be assessed by observing what happened

when the axon was immersed in a large volume of external fluid. Under normal conditions the membrane conductance required about 10 min. to return to its resting value. If recovery is due to absorption of the excess potassium by the axon it should be greatly accelerated by washing the axon with a large volume of normal sea water. This experiment was performed by the 'dipping' method described in the preceding paper (Hodgkin, 1947 b). The axon was looped in a **V** under the polarizing electrode (*A*) and over the adjacent electrodes *C* and *D* (Text-fig. 1). Electrode *B* was not employed, and the conductance was determined from the voltage recorded at the polarizing electrode (V_A).
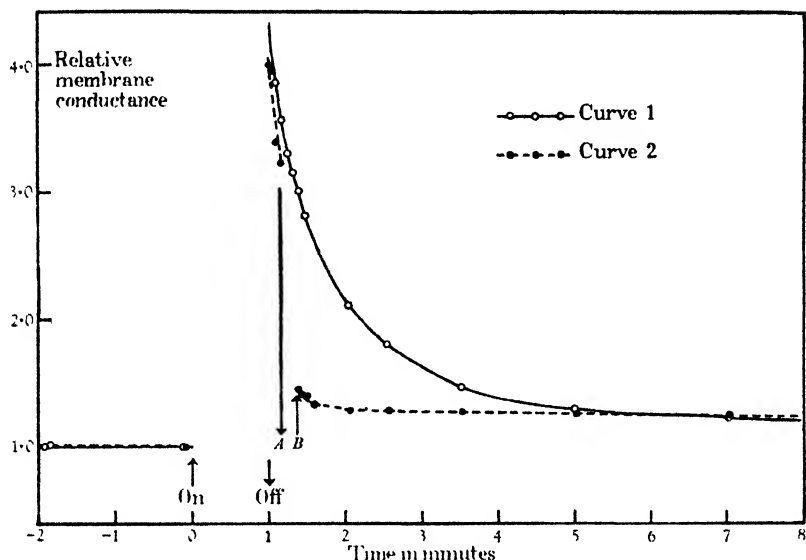
Text-fig. 4. Effect of dipping axon in sea water on time course of recovery. Curve 1, axon in oil throughout; curve 2, axon dipped in sea water between *A* and *B*. Reproduced by permission of the editors of *Nature* from Hodgkin & Huxley (1946).

Curve 1, Text-fig. 4, shows how the membrane conductance was affected by activity when the axon was immersed in oil throughout the experiment. At time 0, a train of impulses of frequency 118/sec. was initiated by connecting the stimulating transformer to the axon. At time 1·0 min. the stimulus was removed and the membrane conductance again measured. By the end of the active period the membrane conductance had increased fourfold, but it returned smoothly to a steady level not very different from that which existed previously. Curve 2 was obtained in exactly the same manner except that the axon was dipped into sea water during the period *AB*. The axon behaved in a similar manner up to the point *A*, but immersion in sea water caused the membrane conductance to return almost immediately to a value which was close to the final recovery level of curve 1. This suggests that the

increase of conductance was largely due to the accumulation of a potassium-like substance in the minute volume of fluid which clings to the axon in oil. Text-fig. 4 suggests that activity may have a small direct effect on the membrane itself, but this change is much less than that due to the potassium effect. The magnitude of the residual effect not removed by sea water was variable, and its magnitude could not be assessed with any certainty. But it rarely amounted to more than about 10 % of the total conductance increment and could therefore be ignored with reasonable safety.

Another way of testing the action of a large volume of sea water was to measure the effect of activity on a section of nerve surrounded by sea water throughout the entire period of the experimental test. The axon could not be totally immersed in sea water, since the membrane conductance would then have been very difficult to measure. But the effect of a large volume could be assessed by immersing part of the axon in the manner shown in Text-fig. 5a. The potential recorded with this arrangement varied inversely with the length exposed to sea water, in the same sort of way that an electrotonic potential changes as the distance between polarizing and recording leads is increased. The recorded potential decreases when the axon is stimulated because the membrane conductance of the parts immersed in oil increases. But the magnitude of the change in potential would be very much greater if the membrane conductance altered uniformly along the axon. A study of the relation between the length of axon immersed in sea water and the magnitude of the change in potential should show whether the axon alters uniformly or whether the increase in membrane conductance is confined to the parts in oil. Application of cable theory to this problem gives the following expression for the effect of a tetanus on the recorded potential:

$$\frac{\text{Potential before tetanus}}{\text{Potential after tetanus}} = \beta_2 \left\{ \frac{(\beta_1/\beta_2 + \alpha)^2 \, e^{\beta_2\theta} - (\beta_1/\beta_2 - \alpha)^2 \, e^{-\beta_2\theta}}{(1+\alpha)^2 \, e^{\theta} - (1-\alpha)^2 \, e^{-\theta}} \right\}, \quad (5)$$

where　β_1^2 is $\dfrac{\text{Membrane conductance in oil after activity}}{\text{Membrane conductance in oil before activity}}$,

β_2^2 is $\dfrac{\text{Membrane conductance in sea water after activity}}{\text{Membrane conductance in sea water before activity}}$,

$\alpha = \sqrt{\dfrac{r_1 + r_2}{r_2}}$,

$\theta = \sqrt{(g_4 r_2)} \times$ length of axon immersed in sea water.

The experiments were made in such a way that equation (5) could be applied directly to the results. The constant α was obtained from measurements of r_1 and r_2 of the kind described by Hodgkin (1947a). A value of 1·82 for β_1 was determined by applying equation (2) to measurements made with the whole axon immersed in oil. The axon was then arranged in the manner shown

in Text-fig. 5a. The length of axon exposed to sea water was varied by raising or lowering the electrode assembly with a screw control. At each length, measurements were obtained of the potentials before and after application of the standard tetanus. The ratio of the two potentials is plotted against distance in Text-fig. 5b. The point at 0·3 mm. was obtained from the change observed when the axon was in contact with the electrode *A* which was

Text-fig. 5 *a*.

MM.
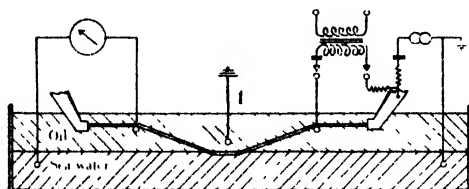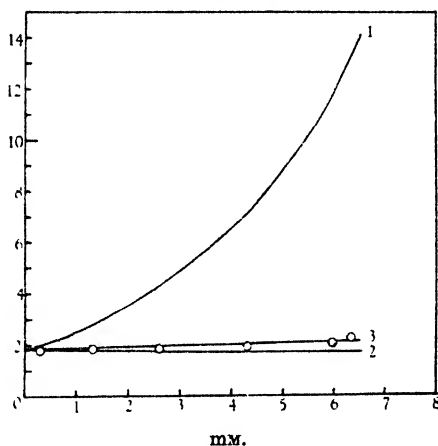
Text-fig. 5 *b*.

Text-fig. 5. *a*. Apparatus used for estimating membrane conductance change in large volume of sea water. *b*. Abscissa: length of nerve immersed in sea water. Ordinate: (Potential recorded before activity/Potential recorded 5 sec. after activity). Activity =7800 impulses in 1 min. Curves 1, 2 and 3 drawn from equation (5) with values of β_1 and β_2 given in text.

0·3 mm. wide. The quantity $(q_4 r_2)^{-\frac{1}{2}}$ is the space constant of the normal axon in sea water and was obtained from the variation of potential with the length exposed to sea water in the normal axon.

Three theoretical curves are plotted in Text-fig. 5b. Curve 1 was calculated on the assumption that the whole nerve changed uniformly so that $\beta_2 = \beta_1 = 1·82$, and curve 2 on the assumption that the membrane conductance did not change in sea water so that $\beta_2 = 1$ and $\beta_1 = 1·82$. Curve 3 was computed on the assumption that the membrane conductance changed to a small extent in sea water, viz. $\beta_2 = 1·075$, $\beta_1 = 1·82$. The third curve fits the experimental data reasonably and shows that the axon exposed to sea water changed its membrane

conductance by $(1\cdot075)^2 = 1\cdot16$, whereas the axon immersed in oil changed by $(1\cdot82)^2 = 3\cdot31$. The membrane-conductance change in sea water is therefore seen to be only about 7 % of the change in oil. Taken in conjunction with the evidence previously described, this experiment proves beyond reasonable doubt that activity is associated with the leakage of a substance which resembles potassium in its action on the nerve membrane.

The effect of variations in the duration and frequency of activity

The amount of potassium lost by an axon should vary with the number of impulses transmitted. An approximately linear relation between the conductance change and the frequency of the stimulus applied is to be expected, since the membrane conductance is roughly proportional to the external concentration of potassium. Text-fig. 6 shows the effects produced on one fibre by a 1 min. tetanus with frequencies varying between 109 and 4·3/sec. The increase in membrane conductance clearly varies in an approximately linear manner with the number of impulses transmitted. There are deviations from a straight line, but there is no reason to suppose that these were not due to random error. Other experiments gave larger deviations, but did not appear to follow any definite pattern, and we are inclined to stress the general proportionality between conductance change and number of impulses rather than the deviations occurring in any particular experiment.

Text-fig. 6. Ordinate: (Membrane conductance 10 sec. after end of activity/Membrane conductance before activity). Abscissa: number of impulses in tetanus lasting 1 min.

However, it would be surprising if the relation were strictly linear, since a result of this kind could only be expected if the following conditions were satisfied: (a) the rate of reabsorption of the potassium-like substance by the axon was negligible or directly proportional to concentration; (b) the amount of potassium lost per impulse was independent of the spacing of the action potentials; (c) the relation between membrane conductance and external potassium concentration was strictly linear.

The cumulative effects of impulses on membrane conductance can be illustrated by sampling the membrane conductance while the tetanus is in progress. The conductance could not be measured during activity (unless the frequency was very low), but it could be estimated by switching off the stimulus for 1 sec. and recording the conductance change at that moment.

An experiment of this kind is illustrated by Text-fig. 7 which shows the effect of 10 min. tetani. When the frequency of the stimulus was 11·3/sec. (curve *A*) the conductance continued to increase during the whole period of activity. After 5 min. the curve rose less steeply and would probably have settled down to a steady level if the stimulus had been maintained for 15–20 min. When the frequency was increased to 52/sec. the curve rose more steeply, but reached its equilibrium value after about 5 min. The fact that the equilibrium value was reached more quickly is explained by the assumption that the rate of reabsorption of potassium varied as the square or cube of the potassium concentration. It will be shown later that the shape of the recovery curve
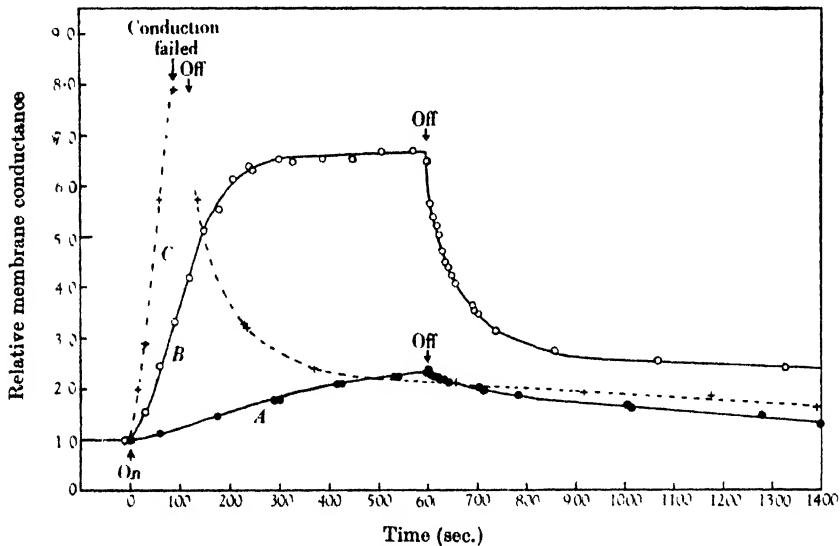
Text-fig. 7. Ordinate: Membrane conductance/Membrane conductance before activity. Abscissa: time in sec. Curve *A* 10 min. tetanus of frequency 11 3/sec.; *B*, 10 min. tetanus of frequency 52/sec.; *C*, 2 min. tetanus of frequency 142/sec.

is consistent with this assumption. When a stimulus of 142/sec. was employed, the membrane conductance rose rapidly to a value of about 8 at which point the axon became inexcitable—presumably because the potassium concentration rose to the point at which it blocked transmission. The stimulus was removed when transmission failed, and the conductance then recovered in a normal manner. Tests made later showed that excitability also returned, although the exact point at which this occurred was not determined.

All three curves show that the conductance started to increase at an accelerating rate. This phenomenon was noticed in many experiments and may be plausibly explained by the fact that the potassium-conductance curve appeared to show an upward curvature when the potassium concentration was small (Hodgkin, 1947*b*).

The magnitude of the potassium leakage per impulse

The amount of potassium which leaks through 1 sq.cm. of membrane during one impulse could be estimated by assuming that the change in membrane conductance was entirely due to an excess of potassium in the external fluid. The experimental measurements were performed in three stages. The first stage consisted of a determination of the volume of external fluid, the second of a measurement of the number of impulses required to raise the external potassium concentration to a known level and the third of an optical measurement of the axon diameter.

The determination of the external volume

Electrical constants of each axon were determined in the manner described by Hodgkin (1947a). The volume of external fluid was obtained from the specific resistance of the sea water used and the value of the external resistance per unit length. The results of these determinations are given in Table 2. The average volume of sea water surrounding 1 cm. length of axon is seen to be 3.15×10^{-6} c.c. This indicates that the equivalent thickness of the saline film outside the axon was approximately 3μ. Optical measurements were made in order to find out if this result was reasonable. Isolated axons are surrounded by a sheath of connective tissue the thickness of which varies between 2 and 5 μ. When such axons are immersed in oil there appears to be no measurable gap between the outside of the connective tissue and the surface of the oil. The photographs in Pl. 1 (which were obtained on an axon with an unusually thin sheath) illustrate this point. Pl. 1 C is a photomicrograph of a stretch of axon immersed in sea water and was obtained with a $\frac{1}{6}$ in. water-immersion objective. Pl. 1 D was obtained on the same stretch of axon immersed in oil with $\frac{1}{7}$ in. oil-immersion objective. The magnification of the two optical systems was different but the photographs in Pl. 1 C, D were enlarged to extents which exactly compensated for the difference. The oil-water interface is clearly visible in Pl. 1 D and may be seen to correspond exactly with the points defined by the outer edges of the connective tissue in Pl. 1 C. This result is to be expected on physical grounds, since the force of surface tension at the curved oil-water interface must exert a pressure of about 1/50 atm. which should be sufficient to prevent appreciable quantities of sea water clinging to the outside of the connective tissue. The external electrolyte space must exist mainly in the interstices of the connective tissue, since there is virtually no fluid outside the sheath. It is not unreasonable to suppose that a fair proportion of the region occupied by connective tissue is in diffusion equilibrium with sea water. The sheath is certainly not an effective barrier to ionic diffusion, since previous work (Hodgkin, 1947b) showed that electrolytes reach the excitable surface within a few seconds of their application to the outside of the sheath.

The absence of sea water outside the connective tissue suggests that the electrically estimated volume should be less than the volume of connective tissue. The latter quantity is not easy to measure, but a rough estimate was obtained in two experiments by determining the thickness of the sheath and the axon diameter at various points along the axon. The following results were obtained:

Volume of sea water outside axon per cm. (electrical estimate)
$$2 \cdot 79 \times 10^{-6} \text{ c.c., } 2 \cdot 62 \times 10^{-6} \text{ c.c.}$$

Volume of connective tissue per cm. (optical estimate)
$$4 \cdot 27 \times 10^{-6} \text{ c.c., } 4 \cdot 52 \times 10^{-6} \text{ c.c.}$$

These figures suggest that about 60 % of the connective tissue is an electrolyte space. This seems to be a very reasonable result and indicates that the electrical estimates probably give the right order of magnitude for the external volume of sea water.

The increase in potassium concentration per impulse

The method of measuring the absolute rise in potassium concentration caused by each impulse was designed to satisfy three criteria: (1) the duration of activity should be so short that appreciable quantities of potassium are not reabsorbed by the axon; (2) the rise in conductance resulting from activity should be compared directly with the effects produced by application of a potassium-rich solution; and (3) the effect of the applied KCl solution should be measured before the axon has absorbed appreciable quantities of potassium from the external fluid. The experimental arrangement was similar to that used in the 'dipping' experiments described on p. 349. The axon was looped in a V under the polarizing electrode (see Hodgkin, 1947*b*, Text-fig. 5) and a continuous record of membrane conductance obtained from the variations of the voltage recorded at the polarizing electrode. Potassium-rich solutions were applied by placing a watch-glass filled with test fluid under the axon and lowering the axon for a few seconds by means of a screw adjustment attached to the electrode assembly. Frequent control dips into sea water were made in order to ensure that the outside of the axon was in equilibrium with sea water and that the membrane was not disturbed by passing through the oil-water interface. A typical result is shown in Text-fig. 8. At the point marked *A* the axon was dipped into sea water for a few seconds: this produced no significant change in conductance and was regarded as a satisfactory control. At *B* a tetanus of frequency 115/sec. was applied to the axon. Samples of membrane conductance were taken at 15 sec. intervals by switching off the stimulus for a period of $\frac{1}{2}$–1 sec. The number of impulses preceding each sample are indicated by the figures attached to each curve. The stimulus was switched off after 1 min. and the membrane allowed to recover. Sea water was applied

at the end of the recovery period and again produced no significant change—suggesting that reabsorption of potassium was complete. The axon was dipped into sea water containing 30 mm-KCl at D for 20 sec. and the rise in conductance measured as soon as the axon had been raised into oil. There was some difficulty in determining the exact magnitude of the initial potassium effect, since the first three points showed a very rapid recovery which may have been spurious. As a compromise, the second point was chosen as giving the effect of 30 mm-KCl. Sea water was applied at E to wash off the excess potassium which had not been absorbed by the axon. The tetanus was repeated at F.
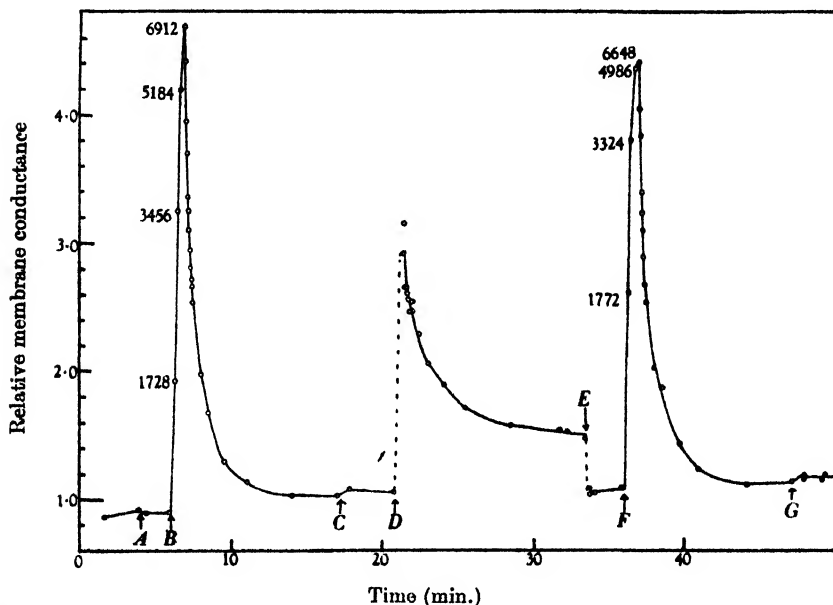
Text-fig. 8. Effects produced by activity and externally applied potassium excess. A, axon dipped into sea water; B, 1 min. tetanus started; C, axon dipped into sea water; D, axon dipped into isotonic sea-water solution containing 30 mm-K; E, axon dipped into sea water; F, 1 min. tetanus started; G, axon dipped into sea water.

The number of impulses equivalent to a concentration of 30 mm-KCl could be assessed from the graph. The first tetanus gave a figure of 2938 and the second one of 2152. In the case of the first tetanus the potassium leakage per impulse per cm. of nerve was therefore

$$\left(\frac{30 - 9 \cdot 8}{2938}\right) \times 10^{-6} \text{ mol./c.c.} \times \text{volume external fluid/cm.} = 1 \cdot 92 \times 10^{-14} \text{ mol.cm.}^{-1},$$

the concentration of potassium in sea water being taken as 9·8 mm. The potassium leakage per sq.cm. of membrane was obtained from this figure and the average axon diameter: in this case it was $2 \cdot 00 \times 10^{-12}$ mol.cm.$^{-2}$

The results of eleven determinations of potassium leakage are given in Table 2. There is considerable scatter in the individual measurements, but they are clearly of the same order of magnitude. Membrane capacities were estimated in some of the experiments and the values obtained are tabulated, since there may be a theoretical relation between capacity and the quantity of potassium lost in each impulse.

TABLE 2. Absolute magnitude of potassium leakage/impulse. (Figures are enclosed in brackets when a single measurement was used more than once.)

Axon diameter (μ.)	Volume of external fluid/cm. (c.c. $\times 10^{-6}$)	Membrane capacity/sq.cm. (μF.)	Potassium leakage impulse/cm. length axon (mol.cm.$^{-1} \times 10^{-14}$)	Potassium leakage/ impulse/cm.^{2} membrane (mol.cm.$^{-2} \times 10^{-12}$)
35·6	3·82	—	1·16	1·03
{34·8	{3·67	{1·03	1·90	1·74
{34·8	{3·67	{1·03	1·37	1·25
{30·5	{3·63	{1·53	1·84	1 92
{30·5	{3·63	{1·53	2·25	2·35
{30·5	3·21	{1·53	2·16	2·26
{30·5	{2 79	{1·53	1·92	2·00
{30·5	{2·79	{1·53	2·62	2·74
{27·8	2·53	0 75	0·81	0·92
{27·8	1·97	2·02	1·00	1·15
30·9	2·89	0·98	1·66	1·72
Average 31·3	3·15	1·35	1·70	1·73

The recovery of membrane conductance after activity

The most plausible explanation of the recovery in membrane conductance is that the potassium lost during activity was reabsorbed from the external fluid by the axis cylinder. Another possibility is that the excess potassium disappeared by diffusing along the axon into the agar wick electrodes which acted as potassium sinks. Calculation indicates that a process of this kind would require about 1 hr. to be 70 % complete. It was therefore unlikely to account for more than a fraction of the recovery observed. The effect of diffusion into the electrode could be eliminated experimentally by removing the polarizing electrode from the axon during the greater part of the recovery period. The electrode had to be brought into contact with the axon for a few seconds in order to determine the recovery curve. But the total time in which it was in contact could be made so small that diffusion could be safely ignored. This test showed that recovery occurred in exactly the same way whether the electrode was in contact with the axon throughout the whole of the recovery period or during a small part of it. Recovery curves obtained in the normal way therefore give a true picture of the absorption of a potassium-like substance into the axis cylinder.

Recovery curves after five tetani of different frequency are given in Text-fig. 9 a. In the interpretation of these curves the membrane conductance may be taken as roughly proportional to the external potassium concentration.

The shape of the curves suggests that the rate of reabsorption of potassium was not proportional to the potassium concentration, but varied as a power greater than the first. Thus the time for half-recovery decreased as the frequency of the tetanus was increased. But for a given potassium concentration the rate does appear to be nearly constant, as is shown by the fact that four curves out of five could be superimposed by lateral shifting along the horizontal axis (Text-fig. 9 *b*). The recovery curve which followed the highest
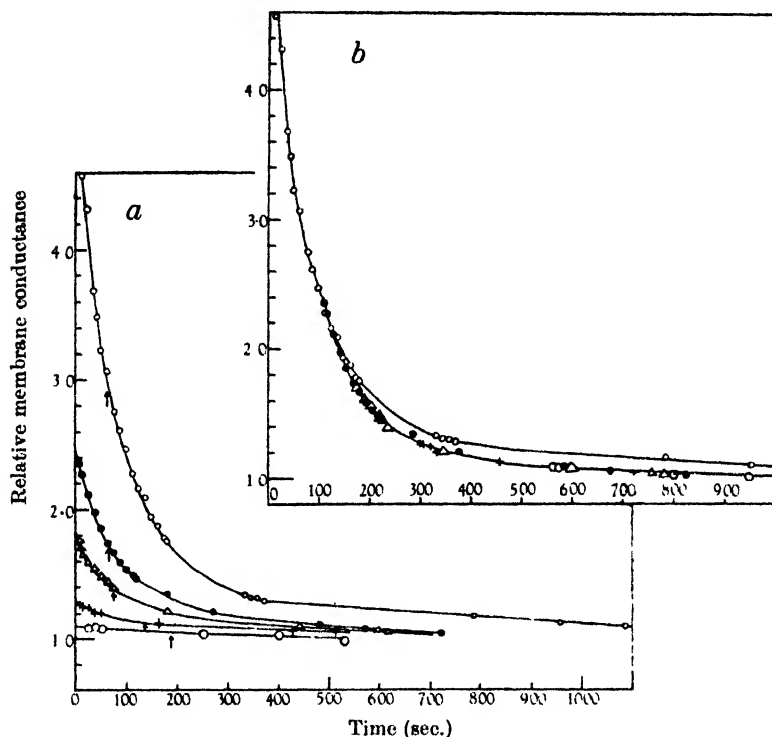
Text-fig. 9. *a*. Ordinate: Membrane conductance/Membrane conductance before activity. Abscissa: time after end of 1 min. tetanus containing the following numbers of impulses: 6552, 2440, 1446, 905, 259. Arrows give the approximate half time of recovery. *b*. Curves of *a* superimposed by lateral shifting along horizontal axis.

frequency tetanus deviates from the others in a manner which suggests that the later stages of recovery proceeded more slowly when the axon had been subjected to a large number of impulses.

Analysis of the curves in Text-fig. 9 indicated that the rate of disappearance of potassium varied as the 1·6th power of the potassium excess. As a rule recovery appeared to increase more rapidly with concentration than this. Table 3 indicates that the average rate of potassium reabsorption at 30 mM-K was about 8 times greater than the value at 20 mM. This suggests that the rate varied as the cube of the excess of potassium.

TABLE 3. Apparent rate of absorption of potassium in $mol.cm.^{-2} sec.^{-1} \times 10^{-10}$.
(Figures in bracketed rows were obtained on same axon.)

After activity		Resting axon	
At apparent K conc. of 30 mM.	At apparent K conc. of 20 mM.	At K conc. of 30 mM.	At apparent K conc. of 20 mM.
0·52	0·16	—	—
(0·75	0·16	—	—
—	—	0·36	0·11
(6·40	0·97	—	—
—	—	1·27	0·13
2·96	0·84	—	—
1·39	0·30	—	—
—	—	0 78	0·15
1·53	0·41	—	—
1·72	0·33	—	—
(3·69	0·13	—	—
—	—	2·95	—
(7·83	0·22	—	—
—	—	0·51	0·10
2·70	0·16	—	—
Average 2·95	0·37	1·18	0·12

The shape of the recovery curve is probably determined by a number of related factors. In the first place there is evidence which suggests that the potassium-conductance relation is not strictly linear but showed an upward curvature. This would tend to make the rate of reabsorption appear faster than it actually was when the potassium concentration was high. A more fundamental reason is that the rate should be determined not only by the 'pressure head' created by the excess of potassium but also by the conductance of the membrane through which the ions are driven. If both membrane conductance and 'pressure head' were proportional to the potassium excess the rate would vary with the square of the potassium excess. A small amount of curvature in the potassium-conductance relation would turn this into a third power law.

The fact that the rate of recovery increased more rapidly than the first power of the potassium concentration explained the rather puzzling fact that the axon appeared to reach a steady state more quickly as the frequency of the stimulus was increased (Text-fig. 7). The argument may be made quantitative by assuming that the rate of return of membrane conductance to its resting value is proportional to the cube of the deviation from its normal value, and that the rate of increase is proportional to the frequency of activity. These assumptions give the following differential equation

$$\frac{dy}{dt} = AF - By^3,$$ (6)

where y is the deviation of membrane conductance from its normal value, A and B are constants and F is the frequency at which impulses are transmitted. Integration of this equation for different values of F leads to a family

of curves which are not unlike those recorded experimentally, as may be seen by comparing Text-figs. 7 and 10.

A curve of the type shown in Text-fig. 8 gives the rate at which the concentration of a potassium-like substance decreases in the external fluid. The absolute rate of transfer through the surface membrane could be calculated from this curve and the estimated values of axon diameter and external volume. The rates of absorption after a tetanus and after the external application of a potassium-rich solution are given in Table 3. The two rates are strictly comparable since they were obtained from successive measurements
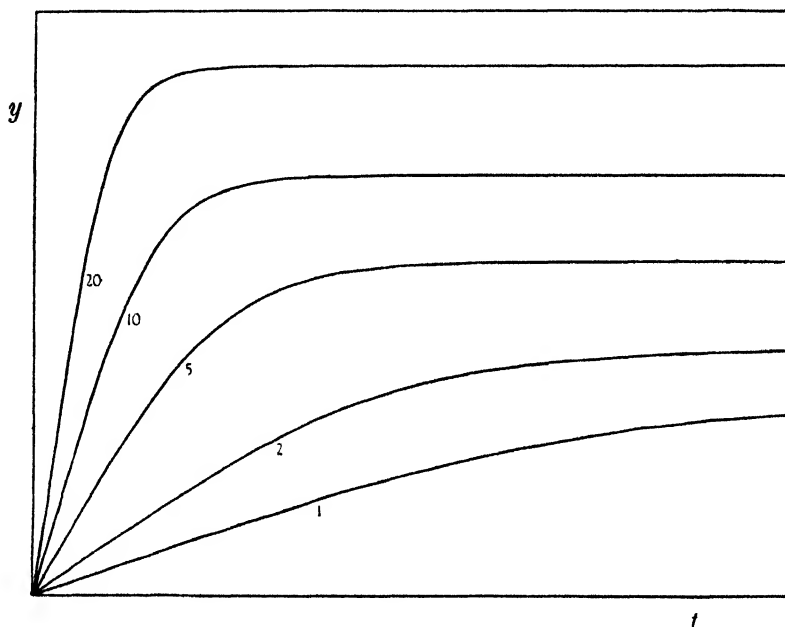
Text-fig. 10. Theoretical curves computed by integrating equation (6) from $y = 0$ at $t = 0$. The number attached to each curve gives the frequency in arbitrary units.

of the kind shown in Text-fig. 8. The average rates of absorption after a tetanus are seen to be about three times greater than the corresponding rates after external application of potassium-rich solutions. Some of this difference must be attributed to inherent defects in the experimental method. In the first place, the polarizing electrode picked up potassium-rich solution which would have delayed recovery when the potassium was applied externally. On the other hand, the polarizing electrode would have acted as a potassium sink when potassium was liberated by the axis cylinder and, if the electrode had any action at all, its effect would have been to hasten recovery. A similar argument applied to any irregularity in the external fluid, since the effect of externally applied KCl would have been to raise the concentration uniformly

along the axon regardless of volume irregularities, whereas the effect of a tetanus was to liberate a fixed quantity of potassium from the axis cylinder. Some of the difference between the two rates of absorption must be attributed to experimental factors of this kind, but a large part of it is thought by us to represent a real difference between the absorption by a resting axon and by one which has been subjected to activity. A physiological explanation of the difference is easily found. Recovery heat production is in full swing for many seconds after a tetanus (Beresina & Feng, 1932), and it would be surprising if some of this energy liberation were not directed towards potassium re-absorption. The potassium ions need not be acted upon directly by a secretory process, since an accelerated rate would be found if sodium or some other internal kation were actively extruded. A more general way of explaining the difference is to attribute it to the large positive after-potential which occurs in many types of axon after a tetanus (Gasser, 1937) and may well exist in crustacean nerve. The inside of the axon becomes more negative than normal during a positive after-potential, and this would tend to suck in potassium ions at a rate which was higher than that in the resting fibre.

According to Table 3 the average rate of potassium absorption after a tetanus was $2 \cdot 95 \times 10^{-10}$ mol.cm.$^{-2}$ sec.$^{-1}$ when the potassium concentration was 30 mM. On the other hand, the average potassium leakage per impulse was $1 \cdot 73 \times 10^{-12}$. These figures suggest that the steady rise in potassium produced by a long tetanus should be 30 mM. when the frequency of activity is $\dfrac{2 \cdot 95 \times 10^{-10}}{1 \cdot 73 \times 10^{-12}} = 170$/sec.

Text-fig. 7 indicates that the steady rise in potassium concentration was of the order of 55 mM. when the frequency was 52/sec. Another experiment gave a steady value of about 25 mM. when the frequency was 31/sec. The shape of curve B in Text-fig. 7 indicates that the discrepancy between these two results and those given in Table 3 was not solely due to the large random errors associated with the absolute measurements. The initial rise of curve B gives a measure of the rate at which potassium is liberated by the tetanus, and the axon must eventually reabsorb potassium at this rate in order to keep the potassium concentration constant during the tetanus. But, when the tetanus was switched off, the conductance fell at about twice the rate at which it rose initially, indicating that reabsorption proceeded more slowly during activity than it did after activity. This result may be attributed to non-linearity in the potassium conductance relation, but a genuine effect of this kind is to be expected on theoretical grounds. For potassium ions cannot be reabsorbed during the action-potential spike, and the reabsorption rate may not reach its full value for many milliseconds after the end of the spike. The rate of potassium reabsorption should therefore be lower while activity is in progress than the rate observed after activity has ceased.

DISCUSSION

The experiments described in this paper prove that activity is associated with the leakage of a substance which affects membrane conductance in a potassium-like manner. The substance leaked has often been assumed to be potassium in order to simplify theoretical arguments and calculations. The evidence for identifying the unknown substance with potassium may now be reviewed. Both unknown substance and potassium caused similar increases in membrane conductance, and both had little effect on membrane capacity. Both were absorbed by the axis cylinder at rates of comparable order of magnitude. The unknown substance appeared to be absorbed faster than potassium, but it may reasonably be supposed that the absorption process operates more rapidly after activity than it does in a resting axon. Another way of approaching the problem is to ask what substance besides potassium might cause a large and reversible increase in membrane conductance. Citrate or calcium-free solutions have been found to raise the membrane conductance (unpublished experiments), but they do so in a manner which is less easily reversible than that of potassium. A further point is that their action is associated with a characteristic instability of the nerve membrane. When citrate is applied to an axon, anodic pulses of the type shown in Pl. 1 A, B are often followed by a sinusoidal oscillation and sometimes by a repetitive discharge (cf. Arvanitaki. 1939). No such action was observed in the present experiments. Substances such as chloroform or alcohol cause irreversible increases in membrane conductance when applied in high concentrations, and it is probable that reversible increases could be produced by application of suitable organic compounds in appropriate concentrations. The possibility that the unknown substance is an organic compound of this type cannot be excluded, but may reasonably be dismissed until evidence in its favour is found. The unknown substance cannot be acetylcholine since this had no action on membrane conductance in *Carcinus* (Hodgkin, 1947 b). A good reason for believing that the substance is potassium is the fact that *Maia* and *Limulus* nerve actually do lose significant amounts of potassium when subject to prolonged stimulation (Cowan, 1934; A. C. Young, 1938). The quantitative estimate given by Cowan is somewhat smaller than ours, but of the same general order of magnitude. Cowan stated that 5.84×10^{-10} moles leak out of 1 g. of nerve during one impulse. J. Z. Young's (1936) histological data for *Maia* suggest that there are roughly 2000 sq.cm. of membrane in 1 g. of nerve (see also Hill, 1932). This indicates a figure of 0.3×10^{-12} for the potassium leakage per sq.cm. membrane which is about one-sixth of our average value. The difference between the two results may be explained by the fact that Cowan's nerves were 'stimulated to fatigue' and many axons may have become inexcitable before the end of the period of stimulation; it is also possible that considerable quantities of potassium may

have been absorbed from the interstitial spaces of the nerve trunk during the 5 min. period of stimulation employed. The identification of the unknown substance with potassium cannot be regarded as proved, in spite of all these considerations. But in the rest of this paper the identity will be assumed without further argument.

The quantity of potassium lost in each impulse may be compared with the number of ions which must be separated by the nerve membrane in order to produce its resting charge. The membrane capacity in *Carcinus* axons is approximately 1.35 μF.cm.$^{-2}$ (Table 2), and the resting membrane potential may be taken as 61 mV. (Curtis & Cole (1942), data on squid axons), so that the charge on the resting membrane must be about 8.2×10^{-8} coulomb cm.$^{-2}$ This suggests that there must be a space charge equivalent to an excess of 8.5×10^{-13} mol.cm.$^{-2}$ of monovalent kation outside the membrane and a deficit of 8.5×10^{-13} mol.cm.$^{-2}$ inside it. The excess and deficit would be largely composed of potassium ions if the membrane permeability to potassium were much greater than that to other ions. The whole of the space charge would have to be neutralized by transport of external sodium or an internal anion before the membrane potential could be reduced to zero. When the membrane repolarizes, the original space charge must be restored by outward migration of a further quantity of potassium ions. These ions would then exchange by diffusion with sodium ions in the external fluid so that the passage of one impulse would result in the loss of the number of potassium ions which carry the resting charge on the membrane. More potassium ions would probably be lost, since the membrane potential reverses during activity and since the action potential has a finite duration. This argument cannot be made rigorous, but it seems to us that potassium leakage could only be an important factor in producing the action potential if the potassium ions lost carried a charge which exceeded that on the resting membrane. Table 2 indicates that the average number of moles of potassium lost in each impulse was 1.73×10^{-12} cm.$^{-2}$ The charge carried by this number of ions is 16.75×10^{-8} coulomb which is about twice the charge on the resting membrane.

The small magnitude of the potassium leakage may be illustrated in several ways. In the first place it may be compared with the total quantity of potassium inside the axon. According to Steinbach & Spiegelman (1943) the concentration of potassium in squid axoplasm may be as high as 360 mM. The concentration in *Carcinus* axons is likely to be less than this, since *Carcinus* blood contains less potassium than squid blood, and the protoplasm of its axons has a higher electrical resistivity (Bear & Schmitt, 1939; Webb, 1940; Cole & Hodgkin, 1939; Hodgkin, 1947a). The concentration (but not necessarily the activity) of potassium ions may be tentatively assumed to be 200 mM. This assumption means that a 35μ axon would lose only 1/100,000 of its internal potassium each time that an impulse travelled along it. The

number of potassium ions lost may also be compared with the number of molecules of fatty acid contained in 1 sq.cm. of a monolayer. If the area occupied by a fatty acid molecule is taken as 20·5 A.^{2} (Adam, 1941), it is found that one potassium ion leaks through an area of membrane which would contain 460 molecules of fatty acid.

The reabsorption of potassium ions after activity can be partly explained without postulating any secretory process if Boyle & Conway's (1941) theory is assumed to apply to *Carcinus* axons. In this case the resting membrane is regarded as permeable to potassium and chloride ions but only slightly permeable to sodium. The theory also postulates that there are considerable quantities of anions in the axoplasm which cannot cross the surface membrane. The following equations would then apply in the equilibrium state:

$$\frac{[K]_2}{[K]_1} = \frac{[Cl]_1}{[Cl]_2} = e^{EF/RT},$$

where [K]$_1$ is the activity of potassium outside the axon and is taken as 9·8 units,

[Cl]$_1$ is the activity of chloride outside the axon and is taken as 537 units,

E is the resting potential and is taken as 61 mV. (Curtis & Cole, 1942),

[K]$_2$ is the activity of potassium inside the axon and equals 110·4 if $E = 61$ mV. at 20° C.,

[Cl]$_2$ is the activity of chloride inside the axon and equals 47·6 if $E = 61$ mV.

Potassium chloride enters the axon if [K]$_1$ [Cl]$_1$ > [K]$_2$ [Cl]$_2$. In the resting axon we assume [K]$_1$ [Cl]$_1$ = [K]$_2$ [Cl]$_2$ = 9·8 × 537. The values for [K]$_2$ and [Cl]$_2$ appear surprisingly low but are not unreasonable since the resistivity of *Carcinus* axoplasm is four times that of sea water.

The simplest way of accounting for the leakage of potassium during activity is to assume that the nerve membrane becomes temporarily permeable to sodium or to one of the internal anions. Only the first possibility will be considered in detail, but it can be shown that the second gives results of a similar type. Two things would happen when the membrane became permeable to sodium: (1) x moles of internal potassium would exchange with x moles of external sodium; (2) y moles of external sodium chloride would enter the axon. The relative magnitudes of the two processes would depend upon the relative permeabilities of the active membrane to potassium and chloride. The product [K]$_1$ [Cl]$_1$ increases from its original value of 9·8 × 537 to $(9·8 + x/\rho_1)(537 - y/\rho_1)$, where ρ_1 is the volume of external fluid. The product [K]$_2$ [Cl]$_2$ increases from 110·4 × 47·6 to $(110·4 - x/\rho_2)(47·6 + y/\rho_2)$, where ρ_2 is the volume of axoplasm water. In these experiments $\rho_2 \doteq 2\rho_1$, so that the calculated increase in [K]$_1$ [Cl]$_1$ exceeds the increase in [K]$_2$ [Cl]$_2$ unless

$y > 7x$ (approximately). After activity, therefore, potassium chloride will tend to enter the axon unless the amount of chloride gained during activity greatly exceeds the amount of potassium lost. A high proportion of the potassium lost is reabsorbed if y is small. If the amount originally lost is sufficient to raise the external concentration threefold, the proportion reabsorbed is 89·4 % if $y = 0$, 78·3 % if $y = x$ and 66·8 % if $y = 2x$. In each case a balance sheet can be made out for the total effect of activity and recovery; for instance, if $y = x$, the net loss to the axon is 0·22x potassium and the net gain is 2x sodium and 1·78x chloride. On the assumption that the membrane became temporarily permeable to an internal anion 89·4 % of the potassium lost would be reabsorbed. The balance sheet is then: loss x mol. anion and 0·11x mol. potassium: gain 0·89x chloride. The first hypothesis indicates that an axon would undergo a minute increase in volume after a period of intense activity, whereas the second suggests that it would shrink by an even smaller amount. In both cases potassium ions would be reabsorbed from the external fluid without the intervention of an active process.

The preceding argument is put forward only because it is the simplest qualitative explanation of the facts. There are several reasons for believing that the true situation is more complicated. The axon could not continue to gain sodium chloride indefinitely, although Steinbach & Spiegelman's (1943) work suggests that it might do so for some time. It is just conceivable that the axon might lose anions continuously and make good the loss by a metabolic process. But such a hypothesis cannot be entertained seriously until more is known about the chemical nature of the internal anions. In either case we are faced with the difficulty that the rate of reabsorption of potassium appears to be faster after activity than in a resting axon. This strongly suggests that a secretory process is at work and is more active after a tetanus. The existence of a positive after-potential would be consistent with this hypothesis. The secretory process need not act directly on the potassium ions, since the same result would be achieved if sodium ions were actively extruded or the resting potential maintained by some unknown mechanism.

One important limitation to our results must be mentioned. In the present work the potassium which leaked from an active fibre was retained in a small volume of external fluid and eventually reached a relatively high concentration. On any view this increase in concentration should assist the process of potassium reabsorption. In living animals potassium leaks into a large volume of fluid so that the external potassium concentration would rise very little as a result of activity. The rate of reabsorption of potassium under natural conditions is therefore likely to be less than that observed in our experiments.

SUMMARY

1. The cumulative effect of a train of impulses on the membrane conductance of an isolated axon from *Carcinus maenas* is described. Axons were immersed in oil and were therefore surrounded only by a minute volume of sea water.

2. The membrane conductance at the end of a short burst of intense activity was found to be three or four times greater than normal. After the activity had ceased, the conductance returned smoothly to its normal value with a half time of a few minutes.

3. Recovery of membrane conductance could be greatly accelerated by washing the axon with sea water.

4. Activity had only a slight cumulative effect on membrane conductance when the axon was immersed in a large volume of sea water.

5. These results indicate (1) that activity was associated with the leakage of a substance which affected membrane conductance in the same way as do potassium ions, and (2) that the potassium-like substance was reabsorbed during the period of recovery.

6. The substance leaking from the active axon changed the membrane capacity much less than the membrane conductance.

7. The increase in the concentration of the potassium-like substance was roughly proportional to the number of impulses transmitted, provided that the duration of activity did not exceed 30–60 sec. The concentration appeared to reach a steady level after a few minutes when activity was maintained for a long time.

8. The quantity of potassium lost by an active axon was calculated on the assumption that the effects observed were due to leakage of potassium. An average value of $1 \cdot 7 \times 10^{-12}$ was obtained for the number of moles of potassium which leak through 1 sq.cm. of membrane during one impulse. The rate at which potassium was reabsorbed appeared to be 3×10^{-10} mol.cm.$^{-2}$ sec.$^{-1}$ when the external potassium concentration was three times its normal value.

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Plate 1
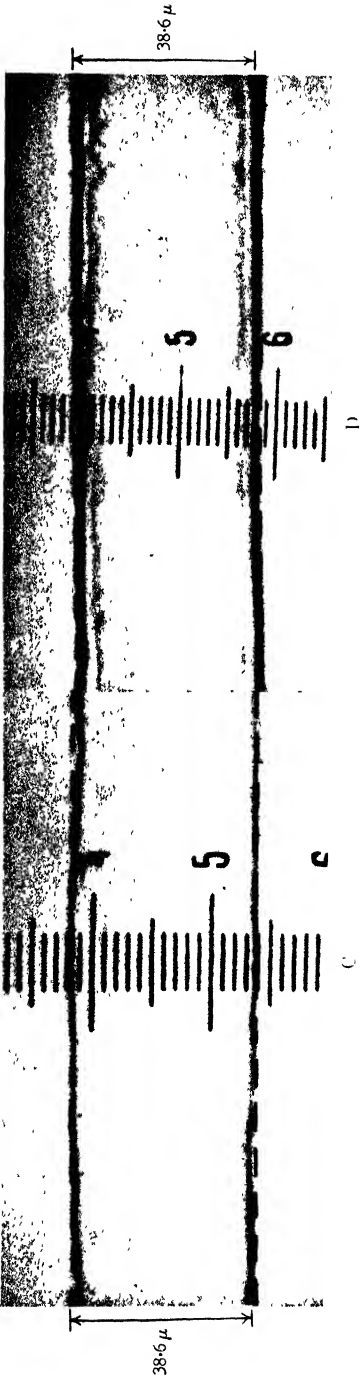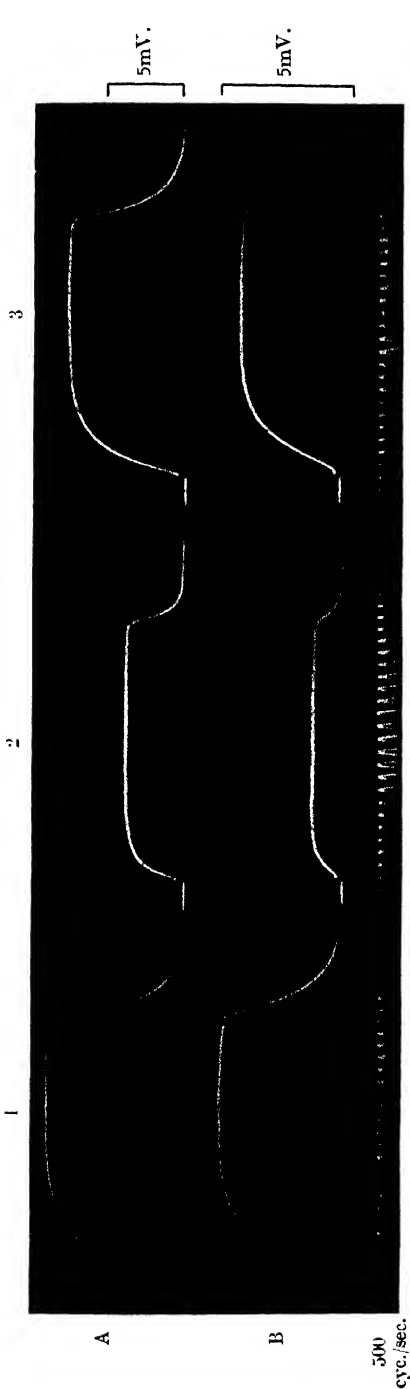
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EXPLANATION OF PLATE I

A. Extrapolar potentials recorded at 1·15 mm. from polarizing electrode.

B. Extrapolar potentials recorded at 2·93 mm. from polarizing electrode.

    1, before stimulation.

    2, 5 sec. after 30 sec. tetanus of frequency about 117/sec.

    3, 13 min. after tetanus

C. Photomicrograph of axon immersed in sea water obtained with $\frac{1}{6}$ in. water-immersion objective and microscopic magnification of 800. 10 small micrometer divisions $= 25\,\mu$.

D. Photomicrograph of same stretch of axon immersed in paraffin oil obtained with $\frac{1}{7}$ in. oil-immersion objective and microscopic magnification of 1000. 10 small micrometer divisions $= 20\,\mu$.

The overall magnification is identical in both photographs. The outer edge of the axis cylinder is barely visible in C. In D it is visible, but refraction at the curved oil-water interface makes the axis cylinder appear too thin.

THE EFFECT OF CHANGE OF BRIGHTNESS LEVEL UPON THE FOVEAL LUMINOSITY CURVE MEASURED WITH SMALL FIELDS

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Wright (1946) has shown in a limited number of experiments that the shape of the foveal luminosity curve, measured with fields subtending small angles (< 20′) at the eye and viewed with the centre of the central fovea, depends upon the particular brightness level at which the measurements are made. The differences of shape were not large, but during determination of the luminosity curves for various areas of the retina within the central fovea in addition to the centre (Thomson & Wright, 1947), similar effects were again found and in the present experiments the luminosity curves for the three foveal positions used by Thomson & Wright (1947) have been obtained at six brightness levels, one of which was the threshold level. It was hoped that the changes observed might throw further light upon the behaviour of the foveal receptor mechanisms.

METHOD

The luminosity curves were measured with the colorimeter developed by Wright (1927, 1939, 1946). In this instrument the longitudinal chromatic aberration of the eye is corrected by the attachment to the eyepiece of a small lens having approximately zero power for yellow light and +3·0 D. chromatic over-correction between 0·700 and 0·400 μ. The step-by-step method of measurement, details of which were reported in a previous paper (Thomson & Wright, 1947), was used and the field size etc. were exactly similar to those in measurements of the 'B' type.

Briefly, a small circular field, whose diameter subtended an angle of 15′ at the eye, was divided horizontally into an upper, test, and lower, comparison, field of equal size. The 'central' curve was obtained by setting the comparison field to wave-length 0·520 μ and adjusting its brightness to one of the levels given in Table 1. The test field of wave-length 0·530 μ was then matched in brightness to the comparison field; the wave-length of the comparison field was then adjusted to 0·530 μ and its own brightness matched to that of the test field (0·530 μ). This done, the wave-length of the test field was altered to 0·540 μ and a further brightness match made. In this way, large colour differences between the test and comparison fields were avoided and the measurements were improved in precision. In any run, observations were taken first towards the red. The luminosity was then calculated from the energy content of the test field.

Since the errors may be cumulative in this method of measurement, some change of brightness level might be expected to occur towards the 'tails' of the curve. The last comparison field towards both red and blue wave-lengths was, therefore, matched to a test field of wave-length 0·520 μ so

as to obtain a measure of the brightness level change, and it was found (if it is accepted that this direct match gives a true estimate of brightness level change) that, both towards red and blue wave-lengths, a rise of brightness of the comparison field occurred at the 'tails' of the curve by a factor of 1·5–2·0.

The 20′ and 40′ curves were obtained in separate experiments by displacing the test field appropriately into the left visual field and equating it in brightness to the comparison field which remained always at the fixation point. The comparison field was adjusted to have an energy content appropriate to both the wave-length and the brightness level under test, and figures obtained from measurements of the central curve were used to make this adjustment, so that the brightness level was maintained throughout the three curves at any one of the six values.

To measure the threshold curve the comparison field was converted into a small red fixation spot which lay immediately below the centre of the flat side of the test semicircle. This spot was adjusted to as low a brightness and to as small a size as was consistent with proper eye fixation. Then at any wave-length the energy content of the test field was first raised so that the field was just visible and then lowered until the field was just invisible and the energy recorded in each case. Two further readings were made and the measurements repeated at the next wave-length. The wave-lengths were taken in order from red to blue and the luminosity for any wave-length was calculated from the mean of five sets of four recordings for that wave-length, each set being obtained on a separate day.

The right eye was used and the results apply to one observer only (L.C.T.). All three retinal image positions lie within the central fovea and the results have, therefore, been assumed to be uninfluenced by the activity of the rod receptors. The energy calibration of the colorimeter did not alter appreciably during the investigation.

Brightness levels. The brightness of the comparison field at wave-length 0·520μ determined the brightness level of the observations. By using a flicker method and a field size of 1° 20′, the brightness of the comparison field at wave-length 0·520μ was matched with a brightness produced by illuminating a magnesium oxide surface with the white standard S_B. The brightness of this

TABLE 1. The energy (erg/sec.), radiating from the comparison field at wave-length 0·520μ, which passed through the exit pupil of the colorimeter for five brightness levels

Brightness level	Energy of comparison field at 0·520μ (erg/sec.)
1	$4·21 \times 10^{-7}$
2	$1·44 \times 10^{-6}$
3	$5·55 \times 10^{-6}$
4	$4·23 \times 10^{-5}$
5	$8·22 \times 10^{-5}$

magnesium oxide surface was then compared to the brightness produced upon a surface of known reflexion factor by a standard lamp (standardized at the National Physical Laboratory). The energy (erg/sec.), radiating from the comparison field at wave-length 0·520μ, which passed through the exit pupil of the colorimeter (diameter 2 mm.) was calculated by using the figure 650 lumens per watt at 0·555μ for the luminous efficiency (Wensel, 1939). The results for the five levels are given in Table 1.

RESULTS

The luminosity curves, which, for each of the three retinal positions and for five of the six brightness levels, are shown in Figs. 1–5, record the logarithm of the reciprocal of the amount of energy at each wave-length required to produce a visual sensation of brightness of constant magnitude. These curves may be regarded as retinal sensitivity curves and the ordinates record the logarithm of the sensitivity, the maximum ordinate of the central curve being adjusted in

each case to 2·00. The difference in magnitude between the ordinates of the central, 20′ and 40′ curves is thus a measure of the difference of sensitivity between the three retinal regions concerned. The curves for level 2 were in every way similar to the sensitivity curves already published for L.C.T. (Thomson & Wright, 1947), and are omitted from the diagrams.
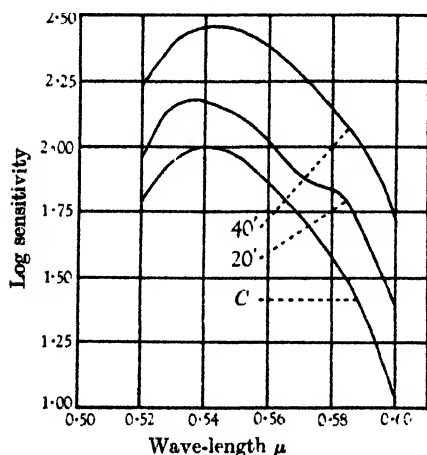
Fig. 1. The sensitivity curves obtained at brightness level 5 for the three retinal positions.
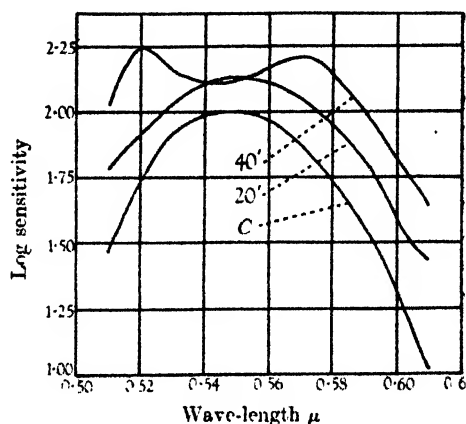
Fig. 2. The sensitivity curves obtained at brigh· ness level 4 for the three retinal positions.
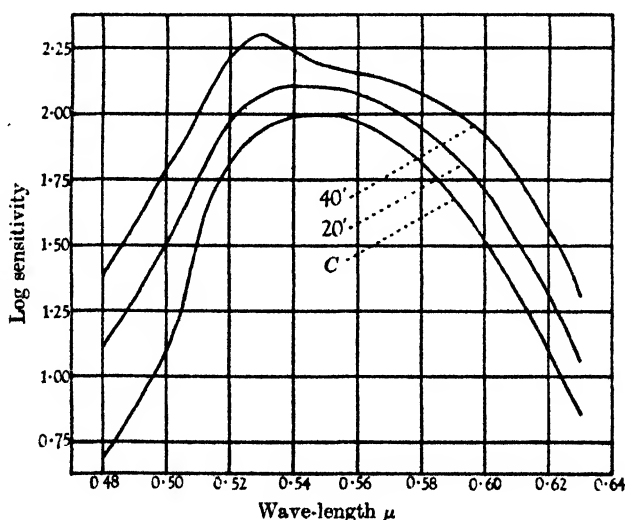
Fig. 3. The sensitivity curves obtained at brightness level 3 for the three retinal positions.

These curves are usually described as the equal-energy luminosity curves and are a measure of the retinal sensitivity at each wave-length. The curves *do not* record the magnitude of the sensation which would be evoked if, at each wave-length, equal amounts of energy were applied to the retina. No measurement

of the magnitude of the response of the receptor mechanisms nor of the magnitude of the sensation aroused by the activity of these mechanisms has been made, but at any level, the set of curves (Fig. 3, for instance) do correspond to a definite amount of receptor response, which, for that level, is the
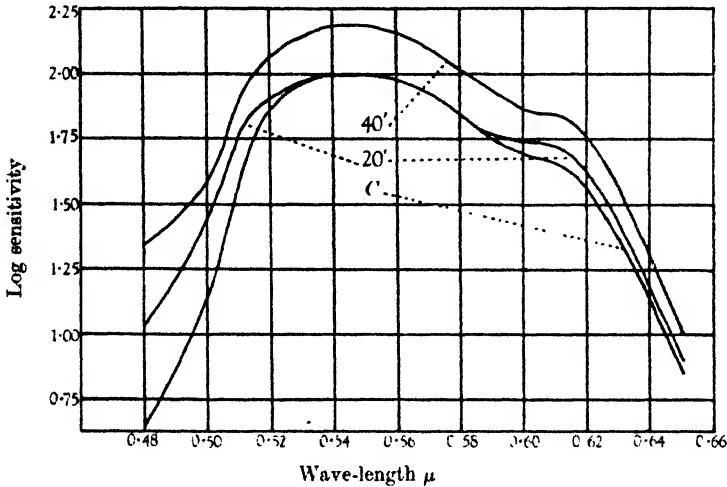
Fig. 4. The sensitivity curves obtained at brightness level 1 for the three retinal positions.
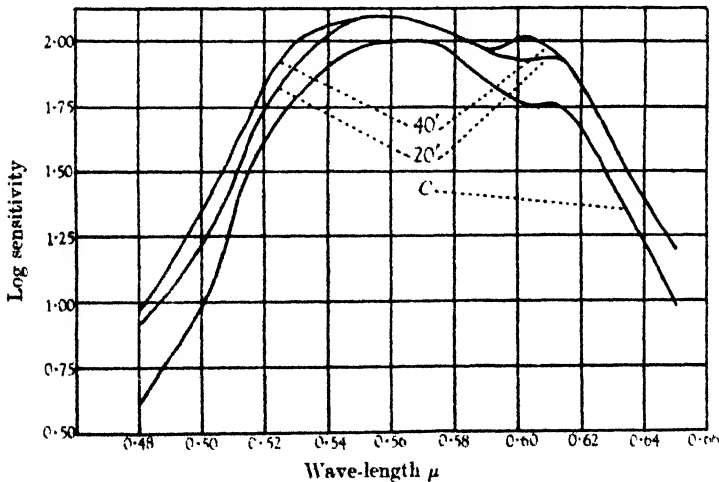
Fig. 5. The sensitivity curves obtained at the threshold level for the three retinal positions.

same for any wave-length and for each of the three retinal areas. The precise magnitude of this amount of response, however, cannot as yet be measured and all one can say is that the response level of Fig. 1 is greater than that of Fig. 2.

The curves were drawn free-hand to be the best fit to the means of five observations at each wave-length. With regard to the accuracy of the results, four typical log standard errors of the mean log sensitivity have been calculated. 'Central' curve: level 3, wave-length $0.520\mu \pm 0.049$; level 2, wave-length $0.600\mu \pm 0.057$. 20' curve: level 4, wave-length $0.520\mu \pm 0.061$. 40' curve: level 4, wave-length $0.520\mu \pm 0.074$. For the central curve the errors were somewhat larger for red than for blue wave-lengths. The central errors did not influence the 20' and 40' readings and the errors in these curves were similar throughout the spectrum. At levels 4 and 5 the errors were somewhat larger than at lower levels.

At level 5 (Fig. 1) the more lateral parts of the central fovea were found to be much more sensitive to light of all wave-lengths than the centre and at wave-length 0.580μ the 20' curve shows a slight hump, the significance of
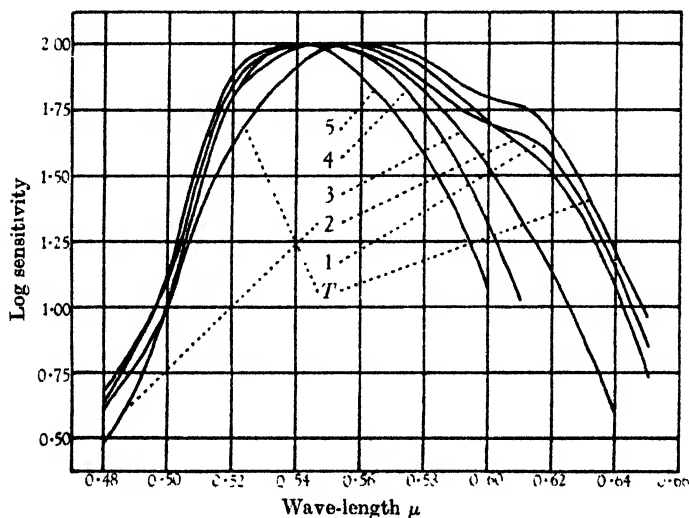
Fig. 6. A comparison of the six luminosity curves obtained from the 'central' position at each of the brightness levels.

which has been assessed by considering the spread of individual readings for both wave-lengths 0.580 and 0.570μ. At level 4 (Fig. 2) the 40' curve shows a marked depression in the green region of the spectrum so that the curve is double-humped with apices at 0.520 and 0.570μ. At level 3 (Fig. 3) the 40' curve shows a similar peak at 0.530μ and large sensitivity changes in the blue region of the spectrum were found. These changes are similar to those reported by Thomson & Wright (1947) and were also present at levels 2 (not given) and 1 (Fig. 4). Level 1 shows further humps in all three curves at wave-length 0.610μ, which are even more pronounced in the threshold curves (Fig. 5).

The six 'central' curves have been compared in Fig. 6 and in this diagram the maximum ordinate of each curve has been adjusted to 2.00.

A striking difference of behaviour between red and blue wave-lengths can be seen. A rise of brightness level resulted in a diminution of the size of red relative to blue ordinates and it will be noticed that the effect is the reverse of

the well-known Purkinje effect. The development of the $0\cdot610\,\mu$ hump at low brightness levels is shown and at level 5, the highest level, the depression of the red ordinates has resulted in a shift towards the blue of the apex of the curve which, at this level, lies at wave-length $0\cdot540\,\mu$.
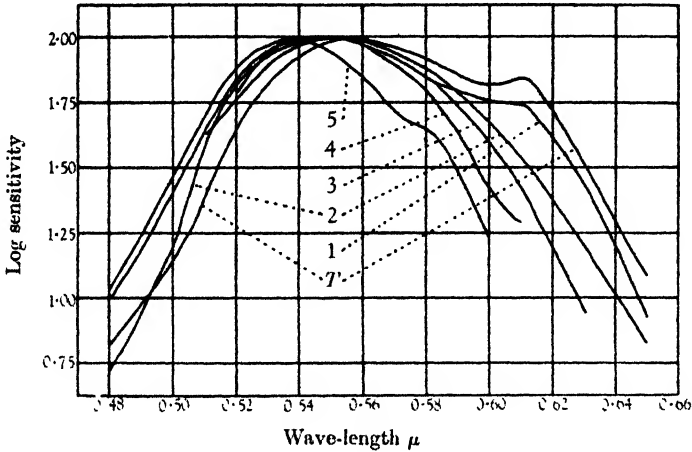
Fig. 7. A comparison of the six luminosity curves obtained from the 20′ position at each of the brightness levels.
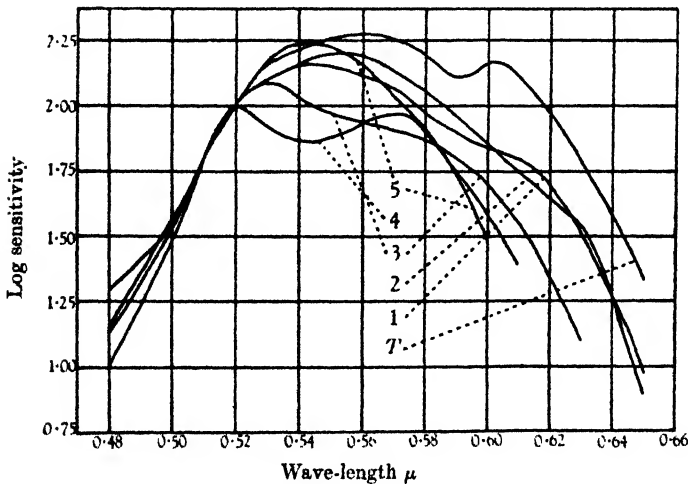
Fig. 8. A comparison of the six luminosity curves obtained from the 40′ position at each of the brightness levels.

It might be argued that this reverse Purkinje effect was in some way linked with the progressive drift of brightness level which may have occurred during the measurement of the curves. It is true that, judged by a direct match between the reddest or bluest comparison field used and a test field of wave-length $0\cdot520\,\mu$, a rise of comparison field brightness did occur towards the

'tails' of the curves, but this rise was, apart from the threshold, in the same proportion for each level and was consistent in all experiments, so that the reverse Purkinje effect is unlikely to be the result of brightness level fluctuations.

If the comparison field was rising slightly in brightness as measurements were carried towards red and blue wave-lengths, this would lead to luminosity values which would be somewhat too low at red and blue wave-lengths and too high at wave-lengths around 0.520μ and the discrepancy seen in Fig. 6 between the threshold curve and that for level 5 is possibly a measure of the magnitude of the drift errors in the step-by-step method.

It might, of course, be said with equal force that the brightness of the comparison field did not change in the step-by-step method and that the direct match was disturbed by the presence of a large colour difference between the two fields. In the discussion below, this view has been adopted.

Fig. 7 records the result of a similar comparison between the 20′ curves and the foveal behaviour in this position is little different from that at the centre.

Fig. 8 shows the comparison of the 40′ curves. Here the ordinate of each curve has been equated at wave-length 0.520μ. Apart from the depression in the green region of the spectrum in levels 3 and 4 the changes are similar to those found at the centre.

DISCUSSION

Hump at 0.610μ. The present experiments show that the hump which is present at wave-length 0.610μ in the curves for all three retinal positions is most pronounced at low brightness levels and this finding confirms that of Wright (1946), except that Wright's hump was at wave-length 0.600μ. A slight bend in the curve at this wave-length, and at low brightness levels, was also noticed by Walters & Wright (1943). The latter were using foveal vision and a rectangular field subtending $2°$ at the eye so that field size appears to be of secondary importance to brightness level in the demonstration of this phenomenon.

Reverse Purkinje effect. The reverse Purkinje effect was, however, not shown in Wright's (1946) investigation. It is possible that the reason for this discrepancy of result is that Wright's test and comparison patches consisted of two small circular fields each 20′ in diameter, whose centres were separated by 25′ of angle, whereas in these experiments the test and comparison fields formed the two halves of one small circular field subtending 15′ at the eye. It is unlikely that the difference was due to the difference between the eyes used, because in a previous investigation (Thomson & Wright, 1947) the two eyes concerned had a closely similar behaviour.

Green depression in the 40′ *curve.* The explanation of this phenomenon is obscure. It is clearly a different formation from the 0.610μ hump, because it

develops only at high brightness levels. The small bend in the 20′ curve for level 5 at wave-length 0·580 μ is probably of the same origin.

It is interesting to note that the 'blue' apex of the 40′ curves at levels 3 and 4 lies at 0·520 and 0·530 μ and that these wave-lengths are within the range of wave-lengths given by Granit (1945) for the apex of the sensitivity curves for the green receptors found by electro-physiological methods in animals.

The energy-response curve. Little is known of the quantitative relationship between the energy applied to the retina in man and the response of the receptor processes; a lack of knowledge which is primarily due to the inability to measure the magnitude of the response. Although no measurements of the
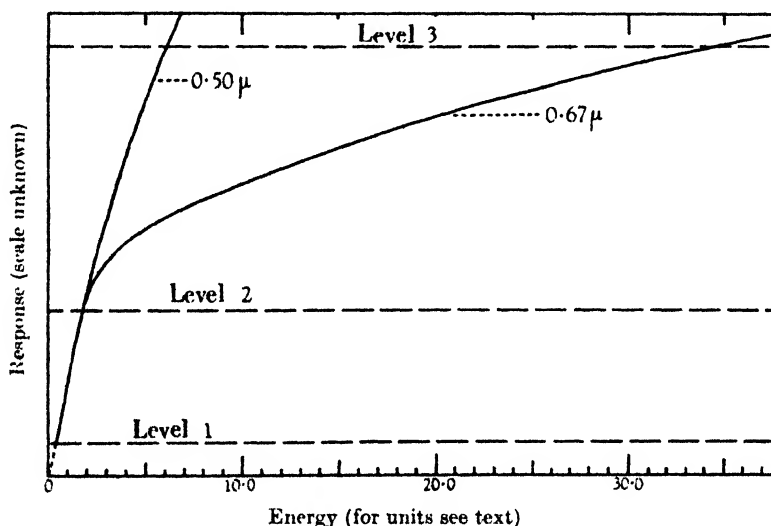
Fig. 9. A summary of the information relating to the energy-response curves for wave-lengths 0·50 and 0·67 μ. The response levels have been marked upon the ordinate at a purely arbitrary position.

size of the response were made, it is possible to draw certain conclusions as to the nature of the energy-response relationship under the present experimental conditions from the curves of Fig. 6. This information is summarized for two wave-lengths, 0·50 and 0·67 μ in Fig. 9 in which it has been assumed that the step-by-step method has yielded a curve, throughout which, for any level, the response at each wave-length is equal. The ordinates in Fig. 9 are, of course, unknown and the response levels have been marked upon it in a purely arbitrary fashion, but the relationship between the levels in terms of energy has been calculated and is correctly given in arbitrary energy units upon the abscissa. To convert these units to erg/sec., 0·4 unit ≡ 2·36 × 10⁻⁶ erg/sec. for wave-length 0·50 μ and 1·26 × 10⁻⁵ erg/sec. for wave-length 0·67 μ. The luminosity curve for level 1 has been used to calculate this relationship. In the

following discussion, if $E_{\lambda.1}$ and $E_{\lambda.2}$ are the energies required at any wave-length λ to reach response levels 1 and 2 respectively, then the 'energy increase' is defined as the ratio $\dfrac{E_{\lambda.2}}{E_{\lambda.1}}$.

The difference of behaviour between red and blue wave-lengths is again well shown in Fig. 9, the retina requiring much larger increases of energy to make a large response step at red wave-lengths than at blue. The relationship in energy between level 1 and the threshold is uncertain for reasons given above, so that the curves have been 'dotted' in this region. Both curves are similar in shape below response level 2, because to make the response step, level 1–level 2, the same energy increase was required at each wave-length. This is indicated in Fig. 6 by the nearly identical shape of the luminosity curve for levels 1 and 2, which was maintained to wave-length 0.670μ.

Both curves cannot be straight lines, because the energy increase required to make the response step, level 1–level 3, was greater for 'red' wave-lengths than it was for 'blue', whilst the increase required to make the step, level 1–level 2, was much the same at all wave-lengths (Fig. 6). One of the curves *could* be a straight line, but a curved form has been preferred for both. It is also clear that the 'red' curve must lie below the 'blue' because the energy increase required to make the larger response steps was always greater for red than for blue wave-lengths. Further, since the reverse Purkinje effect persisted in these experiments to the highest brightness levels, the response curves must continue to diverge up to these levels.

These deductions are quite general and apply to the retina as a unit at the wave-lengths shown. Fig. 9 does not necessarily give the energy response curves for any particular receptor mechanism.

Note added in press.

The yellow human receptor. Whilst this paper was in the hands of the printer, Hartridge (1947) published an explanation of the notch in the luminosity curve which is obtained at low brightness levels, when a small field size is used. He considers that the small depression in the curve which lies to the blue side of the 0.610μ hump (Fig. 6) indicates, under the conditions of small field experiments, an absence of activity of a yellow retinal receptor having a maximum spectral sensitivity at about wave-length 0.580μ. By comparing the luminosity curve obtained by Wright (1946) from that writer's own eye under large field conditions with that obtained by the same worker when using small fields, Hartridge has obtained a spectral sensitivity curve which superficially resembles the sensitivity curve obtained by Granit (1945) for the yellow modulator of the cat's eye.

The results given in this paper point to quite a different conclusion. As may be seen from Fig. 6, the 0.610μ hump is produced at low brightness levels by an increase of the sensitivity of the retina to red, relative to blue wave-lengths and it is difficult to see how absence of activity of a yellow receptor could produce a relative increase of sensitivity at wave-length 0.580μ; indeed, on Hartridge's hypothesis, it is at this wave-length that one would expect such a receptor to produce a maximum decrease in sensitivity.

Fig. 9 shows that on reduction of the light intensity, the response of the retina to red wave-lengths does not fall as sharply as that for blue wave-lengths and because of this relative persistence of response, a receptor mechanism which was mainly sensitive to red wave-lengths might be

expected to cause a hump in the luminosity curve at low brightness levels. Granit's red modulator which has a maximum sensitivity at wave-length 0.600μ could be part of such a mechanism and the 0.610μ hump is best explained by postulating the persistence of activity of a red modulator rather than by the absence of activity of a yellow one.

If the absence of activity of a yellow receptor was the correct explanation of the hump, then the energy-response curve for wave-length 0.580μ would be steeper than that for any other wave-length. For the author's eye this is certainly not the case.

SUMMARY

1. The equal-energy luminosity curves for three retinal positions (central, 20′, 40′), and for six brightness levels have been measured.

2. When the six curves for any one retinal position were compared, a reverse Purkinje effect was found with the development of a hump in the curve at wave-length 0.610μ at low brightness levels.

3. The 40′ curves show a depression in the green region of the spectrum at high brightness levels.

4. The form of the energy-response curve for red and blue wave-lengths is discussed.

5. The yellow human receptor is discussed.

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THE HISTAMINOLYTIC ACTION OF BLOOD DURING PREGNANCY

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Best & McHenry (1930, 1931), confirming the observations of Busson & Kirschbaum (1912) and of Koskowski (1926) find that, even on prolonged incubation, histamine is not destroyed by human blood and only in traces by dog's blood. Their assays of histamine were, however, made on the blood pressure of atropinized cats, a method not allowing of great precision. In complete contradiction to these observations Marcou (1938, 1939), using Barsoum & Gaddum's method (1935), claims that normal blood, plasma and serum rapidly inactivate considerable amounts of histamine. This effect was ascribed to some histaminase being normally present in the circulating blood. The histaminolytic power of blood is expressed by Marcou by an arbitrary histaminolytic index 'HI', which represents the percentage of histamine destroyed in 30 min. at 37° C. by blood to which 3 μg. of histamine diphosphate had been added per c.c. According to Marcou the 'HI' of normal human blood is 30–50 %; of dog's blood, 20–80 %, venous blood destroying more histamine than arterial blood. In allergic conditions the 'HI' is greatly diminished, while, in the febrile state, and in women during labour, it is increased above normal. It is also claimed that the 'HI' conspicuously increases after repeated injections of histamine. On the basis of these observations Marcou makes a number of theoretical deductions concerning the normal metabolism of histamine and the use of histamine in the treatment of allergy.

On looking through the protocols of our published experiments upon the partition of histamine between plasma and corpuscles (Anrep, Barsoum, Talaat & Wieninger, 1938) we found that Marcou's results are not in agreement with our own. In view of this we reinvestigated the problem in detail and with greater precision. The main results of our investigation were published in a preliminary communication in 1941 (Anrep, Barsoum, Ibrahim & Amin, 1941). Since then, our work has been considerably extended. Being cut off during the war from almost all the physiological literature, we were not aware that, meanwhile, the subject had attracted the attention of several other observers and that a number of papers dealing with the same problem had been

published. A full review of this literature, with the exception of our paper, is given by Ahlmark (1944) who, probably owing to the same causes, was unable to obtain our publication, except as an abstract (Chemical Abstracts, 1943). In view of the fact that most of this literature had appeared after the termination of our work a comparison of our results with those of the other observers will be made in the discussion, after the description of our own experiments.

METHODS

The 'HI' was determined (a) by a method similar to that used by Marcou and (b) by a simplified method which, without sacrifice in accuracy, considerably shortened the procedure of the assay.

(a) Serum, plasma or blood, in a volume of 0·9 c.c., or of any multiple of this amount up to 4·5 c.c., is warmed in a thermostat to 37° C. Heparin, or a trace of chlorazol fast pink, are used as anticoagulants. Excess of the latter should be avoided as it somewhat inhibits histaminolysis. A solution of 30 μg. of histamine diphosphate in 1 c.c. of Tyrode's solution is added in proportion of 0·1 c.c. to each 0·9 c.c. of the sample and the mixture is incubated for 30 min. The sample is then treated with 5 or 10 c.c. of a 10% solution of trichloroacetic acid and an extract is prepared by the method of Barsoum & Gaddum (1935) for blood and by Code's method (1937) for plasma and serum. The assay is made on the atropinized guinea-pig's ileum against a standard solution of 0·5 μg. histamine diphosphate per c.c. A second extract is prepared without incubation to serve as a control, the same amount of histamine being added to the trichloroacetic acid solution instead of to the blood. The determination of the histamine content of human blood before the addition of histamine is omitted since, in the presence of 3 μg./c.c. of the added histamine, this would constitute, except in rare cases, only 2-3% of the total, an amount which is beyond the range of accuracy of the method.

(b) In the simplified method the 'active' and the control samples are assayed without preliminary extraction. For the control, 0·9 to 4·5 c.c. of serum are diluted, depending on the volume of the sample, with 5·0 to 25·0 c.c. of Tyrode's solution, and the temperature of the mixture is rapidly raised to over 80° C. After cooling, a solution of 30 μg./c.c. of histamine diphosphate is added in proportion of 0·1 c.c. to each 0·9 c.c. of the serum to make a final concentration of 0·5 μg./c.c. The control sample is then incubated at 37° C. for the same length of time as the 'active' sample.

For the 'active' sample, an equal volume of serum is previously warmed in a thermostat to 37° C., the same amount of histamine is added and the mixture is incubated for exactly 30 min. The sample is then rapidly diluted, depending on its volume, with 5·0–25·0 c.c. of Tyrode's solution and at once heated to 80° C. to arrest further histaminolysis.

The two samples are assayed on the ileum against each other and against a standard solution of histamine. To facilitate the assay when the 'HI' exceeds 95%, the final dilution of the incubated sera was reduced to a half. Serum samples, in which the destruction of histamine is doubtful, i.e. less than 10%, were incubated for 1–4 hr. instead of 30 min. The index was, however, always calculated in terms of the percentage of histamine diphosphate destroyed in 30 min., the initial concentration of the salt being 3 μg./c.c.

The following observations justify the use of the direct method of assay in the simplified form described above. It is well known that blood contains substances which affect the isolated intestine and which render it impossible to make a direct assay of histamine by comparing the action of blood with that of a standard solution of histamine. In presence of such small concentrations of blood as 1% the sensitivity of the intestine to histamine is diminished and its contractions become erratic. Although the action of serum is, in this respect, less detrimental than that of whole blood, nevertheless, accurate estimations of histamine cannot be made in the presence of untreated serum.

After heating to 80° C. the interfering action of human serum and of most animal sera is abolished, histaminolysis is completely arrested while the histamine content of the serum is not affected (Fig. 1).

It can be seen from Fig. 1a that histamine diphosphate dissolved in Tyrode's solution and in human serum, which was then heated to 80° C., evokes identical contractions of the ileum. Fig. 1b shows that in the presence of non-heated serum histamine evokes a smaller contraction and that the sensitivity of the ileum becomes temporarily diminished.

Administration of heated diluted serum, in amounts up to 1 c.c., has, with rare exceptions, no action on the guinea-pig's ileum suspended in a bath 5 c.c. in capacity. Most assays can therefore be satisfactorily made against a single standard solution of histamine in Tyrode's solution. However, since some sera, especially those of herbivora, caused a small relaxation or contraction of the atropinized ileum, all the assays were controlled against separate standards freshly prepared in the diluted and heated serum of each sample.
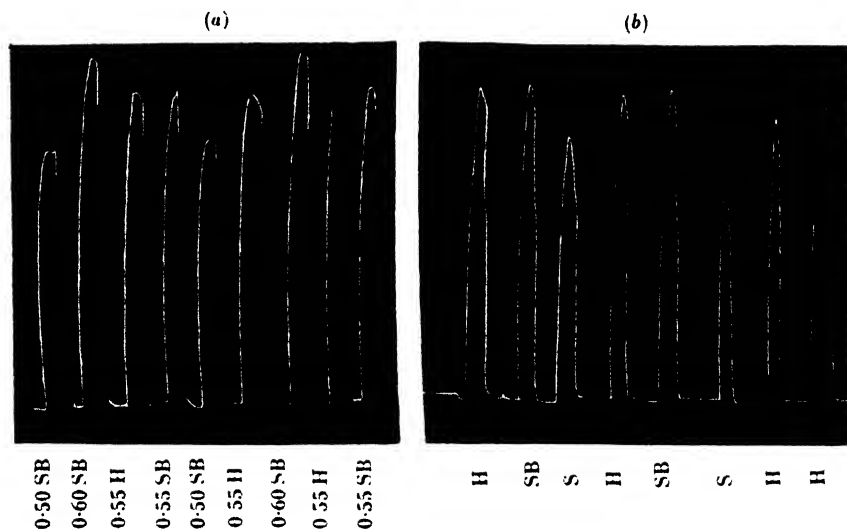
Fig. 1. Direct assay of histamine in heated diluted serum. Guinea-pig's ileum in atropinized (0.5×10^{-6}) Tyrode's solution. Volume of the bath, 5 c.c. (a) Human serum; SB, serum diluted with two volumes of Tyrode's solution; 0·1 μg. of histamine diphosphate was added per c.c. and the mixture heated to 80° C.; H, standard solution of histamine diphosphate, 0·1 μg./c.c. The amounts of the solutions administered to the bath containing the ileum are shown under each contraction. (b) Pig's serum; SB, serum diluted with three volumes of Tyrode's solution and heated to 80° C.; S, the same serum diluted as above but not heated; 0·5 μg. of histamine diphosphate per c.c. was added to the heated and to the un-heated serum; H, standard solution of histamine diphosphate, 0·5 μg./c.c. The three solutions were administered in volumes of 0·2 c.c. each, equivalent to 0·1 μg. of histamine diphosphate. Assays a and b were made on two different preparations of the ileum.

The simplified method is not inferior in accuracy to the method of extraction. It is time saving and the whole assay, including the incubation, can be completed in about 45 min. instead of in several hours. The method of direct assay cannot be used for whole blood since red blood cells contain material which even after heating to 80° C. causes contraction of the intestine and a diminution of its sensitivity to histamine.

As a routine, all histamine determinations were made in triplicate, frequently by two observers working on different preparations of the ileum. The assays were made with histamine concentrations which caused an intestinal contraction between 30% and 50% of the maximal response. Even with these precautions the accuracy of the assays could not be relied upon below a limit of

25—2

10%. Thus, the difference between a histamine inactivation of 90 and 92% (the undestroyed histamine being 10 and 8% respectively) could be reliably determined, while the difference between 0% and 10% inactivation was too small to be detected with certainty.

<div align="center">RESULTS</div>

Histaminolysis in normal blood. The results of over 150 attempts to demonstrate histaminolysis in normal human blood, plasma or serum were completely negative. Whether the method of extraction or of direct assay was used, the histamine values before and after incubation were, within the limits of accuracy of the method, identical. Prolongation of the incubation to 4 hr. did not change the result. In addition, we analysed blood from 27 tubercular patients in different stages of the disease, from 9 cases of cancer or of sarcoma, from 22 cases of different types of allergy, from several cases of advanced congestive heart failure, of pleurisy and of various febrile conditions infective in origin. In all these, as in the normal individual, no histaminolysis could be detected. Blood collected during menstruation behaved like normal blood and did not destroy histamine.

Amongst the usual laboratory and domestic animals no histaminolysis was found in the blood and serum of the dog (over 100 estimations), of the cat (16), of the rat (12) and of the horse (5), the figures in brackets indicating the number of animals used. Absence of histaminolysis was confirmed in each case by prolonging the incubation to 2 hr. Doubtful histaminolysis (i.e. 'HI' $=5-10\%$) was found in the guinea-pig (25), in the goat (6) and in the water buffalo (6). Indices ranging between 'HI' 15% and 'HI' 30% were found in the serum of the pig (5), of the camel (4), of the sheep (6) and of the rabbit (8). In experiments on the dog no difference could be detected between the arterial and the venous blood.

Effect of injection of histamine. Subcutaneous injections of histamine with gradually increasing doses were made in dogs and in three human subjects. It was hoped that this treatment would, as suggested by Marcou, stimulate histaminolysis. In the dog, daily injections of histamine diphosphate were begun with doses of 0·1–0·5 mg. In two animals a maximal dose of 10 mg. was reached in 10 days and continued for another 10 days. In two other animals, the maximal dose was 50 mg./day. In man, the injections began with 0·1 mg. gradually increasing the dose to 1·0 mg. in two subjects and to 3·0 mg. in the third subject. The maximal dose was maintained in each case for 15 days. As a result of this treatment the histamine content of the blood of the injected dogs conspicuously increased above normal, but no histaminolysis could be demonstrated in blood samples taken during, and for some time after the end of the injections. In man, the results were also consistently negative.

Histaminolysis during pregnancy. Marcou reports that just before and during labour the 'HI' of the maternal blood increased from 40–50%, i.e. from a level which he regards as normal, to 60–95%. Marcou considers this increase to be

a defence reaction of the organism against the imminent trauma of labour. We are able to confirm the appearance of a strong histaminolytic agent in the blood during pregnancy. Since, however, according to our observations, no histaminolysis can be detected in normal human blood, its presence during pregnancy becomes much more significant than is indicated by Marcou's figures. Furthermore, we find that the increase of the 'HI' is in no way related, as suggested by Marcou, to the time of the delivery but is progressive, beginning from about the end of the second month of pregnancy, continuing throughout the period of gestation, and reaching a maximum towards term.

Altogether 231 determinations of the 'HI' were made in 136 cases of normal pregnancy; 203 determinations were made in the serum and 28 in whole blood. The simplified method was used for most assays but many were also made with the method of extraction or with both. Most determinations were made immediately after the collection of the blood. However, it was soon found that the serum as well as the blood could be safely kept overnight in the ice chest and, in the winter, even at room temperature (14–24° C.) without diminution of the 'HI'.

The results of the determinations for each month of pregnancy are given in Fig. 2; of the 203 assays on the serum 184 (not shown on the figure) fall within the space between the two outside lines of the figure; 5 (marked by points) are below and 4 above these lines. Of the 28 assays on whole blood (marked by crosses) 12 are below, 8 on the lower line and 8 between the two limiting lines. It follows that as many as 40 % of the 'HI' determinations for whole blood and only about 2 % of those for the serum fall below the expected results.

The middle line of Fig. 2 gives the means for the serum calculated for each month of pregnancy. The figures obtained for whole blood, shown by crosses, are not considered in the calculations of the means. A statistical treatment of all the results obtained with the sera is given in Table 1.

It could be questioned whether the method used can be relied upon for the estimation of the high 'HI' values observed towards the end of pregnancy. The initial concentration of the substrate in the incubated samples, $3 \mu g./c.c.$, may be too small in relation to the amount of the active agent, or the duration of the incubation too long for reliable results. It must be remembered that the 'HI' is an entirely arbitrary unit which is convenient for comparative purposes but has no claim to any absolute value. The following test shows that the conditions selected for the determination of the 'HI' are satisfactory. Four different dilutions of a 9-months-pregnancy serum were made; after addition of histamine to give $3 \mu g.$ histamine diphosphate in each c.c. of diluted mixture, the samples were incubated at 37° C. The rate of histaminolysis observed in the four dilutions is shown in Fig. 3.

It can be seen that, after 30 min. incubation, the 'HI' for the four dilutions was 94, 69, 51 and 34 % from A to D respectively. Dilution A was the same as

used for all the routine estimations described in this communication. It follows from the results shown in Fig. 3 that prolongation of the incubation of dilution *A* would be superfluous, while a diminution of the relative amount of serum would unnecessarily extend the incubation time without much advantage.
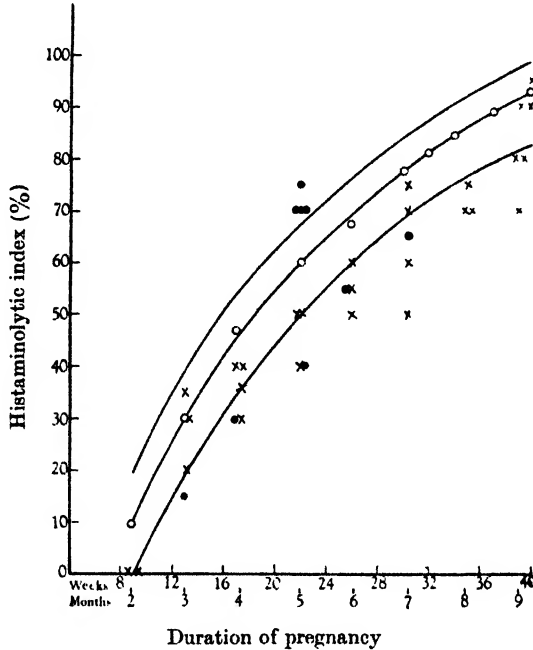
Fig. 2. Histaminolytic index in relation to the duration of pregnancy. The two outside lines enclose 203 observations made on the serum (the individual estimations are not shown); 5 observations for the serum (points) fall below, and 4 above the two limiting lines. The middle line (circles) shows the means of all the observations made on the serum. The 'HI' for whole blood (crosses) is not considered in the calculations of the means. It will be noticed that almost all the crosses fall below the corresponding means. The number of observations for each month of pregnancy is given in Table 1.

TABLE 1. Histaminolysis during pregnancy. The standard error is calculated from the formula $s/\sqrt{n}$, where s is the standard deviation and n the number of observations

Duration of pregnancy		No. of observations	Mean 'HI' %	S.E.
In months	In weeks			
<2	—	20	0·0	—
2	9	21	8·8	±1·87
3	13	15	30·0	±1·89
4	—	12	47·5	±1·93
5	—	14	60·0	±2·34
6	26	13	67·0	±1·67
7	—	11	77·3	±1·97
7½	—	8	81·2	±1·73
8	—	15	85·0	±0·94
8½	—	12	88·0	±1·74
9	39—40	64	92·0	±0·79

Nevertheless, as a safeguard against the possibility of the inactivation being completed before the expiry of the 30 min. period of incubation, all serum samples with an 'HI' above 90% were assayed twice, after 25 min. and after 30 min. of incubation. No cases of normal pregnancy were, however, encountered in which the histamine inactivation reached 90% within 25 min. of incubation.
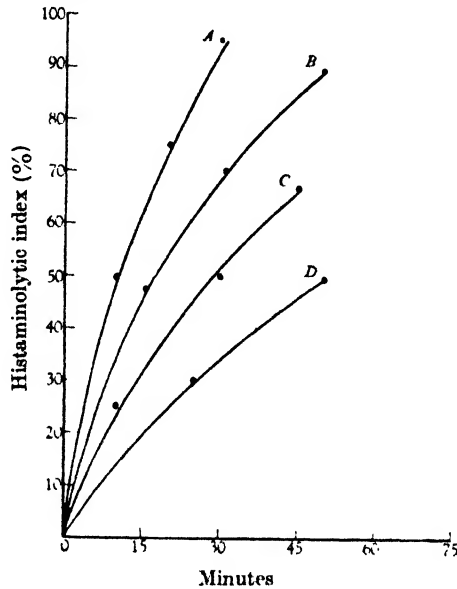
Fig. 3. Rate of histaminolysis in different dilutions of a 9-months-pregnancy serum. The initial concentration of histamine diphosphate was in all samples $3 \mu g$ /c.c. Sample A contained 9 c.c. of pregnancy serum and 1 c.c. of a solution of histamine diphosphate containing $30 \mu g./c.c.$ In samples B, C and D the pregnancy serum was diluted with a normal serum, so that in B, 50% was pregnancy serum, in C, 25% was pregnancy serum and in D, 12.5% was pregnancy serum; 9 c.c. of each dilution were mixed with 1 c.c. of the stock histamine solution. All the dilutions were incubated at 37° C. The concentration of the substrate and of the pregnancy serum in A was the same as that used for the routine estimations of the 'HI'.

Histaminolysis post partum. There is no change in the 'HI' during the first and second stages of labour; it remains at the maximal level reached before delivery. Neither could any change be detected in samples collected 2–4 hr. after delivery of the placenta. A definite diminution does not occur before 8–9 hr. post partum after which the 'HI' declines very rapidly (Fig. 4); 24 hr. after delivery it drops to about 45% and after 48 hr. to 10–20%. Towards the end of the 3rd day it is usually too small for reliable estimation. In a few subjects the 'HI' disappeared on the 4th day, and in two some suggestion of histaminolysis was present as late as the 5th day. It is difficult to determine the exact moment of complete disappearance of histaminolysis with certainty since the smaller the 'HI' the more unreliable is its determination. The important feature of the change is the extreme rapidity with which the 'HI'

of the maternal blood declines after delivery. As an average, at the end of the first day it is reduced to about a half of its maximal value, and at the end of the 2nd day to less than a quarter.

Distribution of the 'HI' in the blood. Marcou reports that blood corpuscles have a lower 'HI' than the serum. It is likely that the separation in his experiments was not complete. We find that, when 9-months-pregnancy blood is centrifuged for 30 min. at 6000–8000 r.p.m., the corpuscles collected from the bottom of the centrifuge tube have no power to destroy histamine even though the serum may have an 'HI' as high as 95 %. In these observations the
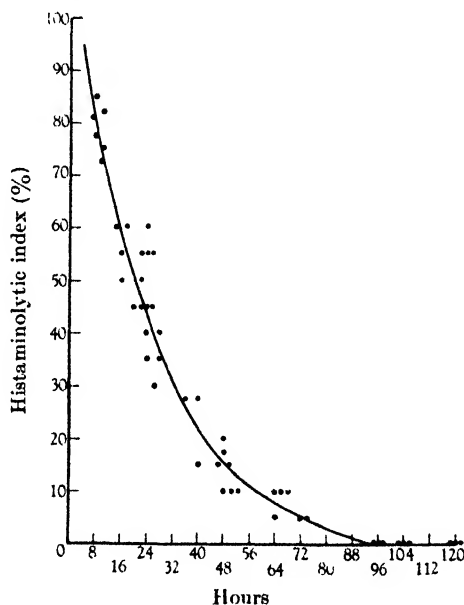
Fig. 4. Diminution of the 'HI' of the serum post partum. Values of 'HI' below 10 % are approximate, indicating a trace of histaminolysis.

red cells were suspended in Tyrode's solution or in the serum of a non-pregnant subject of the same blood group. Histamine diphosphate was then added to give a concentration of $3.0\,\mu$g./c.c. and the mixture incubated for 30 or 60 min. The difference between the incubated and the control samples was in every case well within the limits of accuracy of the method. Preliminary laking of the corpuscles does not change the result.

These observations explain why the 'HI' of whole blood is frequently lower than that of the corresponding serum. The difference may be sometimes considerable. For example, a blood sample collected towards the end of pregnancy had an 'HI' of 80 %, the corresponding serum 95 %, and the corpuscles an index too small to be determined. Similar examples can also be seen in Fig. 2 where most of the 'HI' for whole blood are lower than those for the sera.

In addition to the above, there is another reason why the 'HI' of whole blood may be lower than that of serum, namely, histamine in the corpuscles is somewhat less rapidly destroyed than is histamine in the serum. Anrep *et al.* (1938) found that histamine added to blood distributes itself equally between serum and corpuscles and that, when histamine-rich corpuscles are suspended in normal serum, they retain their histamine, giving hardly any to the serum. This observation enabled us to study the action of pregnancy serum on histamine-rich corpuscles and to compare the result with histaminolysis in the serum. Normal corpuscles and serum, each containing 3 μg./c.c. of histamine diphosphate, were incubated for 30 min. with equal amounts of 9-months-pregnancy serum of the same blood group. In 6 observations the average destruction of histamine in corpuscles was 22 % (s.e. $\pm 3\cdot2$) less than the destruction of histamine in serum.

The source of the histaminolytic agent. The rapid decline of the 'HI' after delivery suggests that the histaminolytic agent, which is presumably identical with histaminase, originates in the foetus or in its appendages. To find the part played by the foetus, blood was collected from the umbilical cord and the 'HI' of the serum was determined in the usual way. In some cases the blood was collected from the placental and also from the infant's end of the cord. Samples of the mother's blood were taken at the same time. The 'HI' of the infant's blood (serum) was in every case much lower than that of the mother, ranging between 25 and 33 % as compared with about 95 % in the mother's blood. The blood from the placental end of the cord had a somewhat higher index than that from the infant's end, but in no case was the difference significant. The low 'HI' of foetal blood makes it unlikely that the foetus serves as a source of the histaminolytic agent. This conclusion is strengthened by the fact that the 'HI' of the child's blood diminishes after birth even more rapidly than that of the mother. Blood samples collected from the fontanella of the child 2–3 hr. after birth had an 'HI' of 5–10 %, a result which is at the limit of accuracy of the method. The 'HI' of the mother is at that time still at its maximum.

As regards the placenta, Marcou reports a single observation in which he found, in a freshly prepared extract, an index of 84 %. He did not pursue the investigation any further. For our observations, pieces of about 5 g. of placenta were compressed between filter paper to remove as much blood as possible. The remaining tissue was weighed and then ground for about 30 min. in a mortar with silver sand and saline, 1·0 c.c./g. of tissue. The fine suspension of the placenta was used for the determination of the 'HI'. The extracts must be prepared as soon after the delivery of the placenta as possible; keeping the organ overnight, even at low temperature. diminishes its histaminolytic action. Care must also be taken to avoid disinfectants such as 'Dettol', etc., since in their presence histaminolysis is greatly diminished or even completely checked.

Most placentae (27) were obtained after normal labour, 2 after Caesarean section, and 8 after miscarriages which took place at different months of gestation. The earliest placenta, from a case of $3\frac{1}{2}$ months pregnancy, weighed 37 g., the next of 5 months 162 g., two of $5\frac{1}{2}$ months 197 g. and 210 g., two of 6 months 250 and 290 g., $6\frac{1}{2}$ months 330 g. and 8 months 425 g. The 29 full-term placentae weighed between 490 and 680 g.

The study of histaminolysis in the placenta led to two important results. First, it was found that the histaminolytic action of all placental extracts was much superior to that of the 9-months-pregnancy serum and, secondly, that in contrast to the results with serum, no relation existed between the power of placental extracts to inactivate histamine and the duration of the pregnancy or the weight of the organ.

In view of the very strong histaminolytic action of placental extracts only small fractions of a c.c. were used for the incubation with histamine diphosphate. We found that 0·15–0·20 c.c. of the extract (equivalent to 0·075–0·1 g. of tissue) had approximately the same histaminolytic action as 1·0 c.c. of serum in the last month of pregnancy. Taking the average 'HI' of 9-months-pregnancy serum as 92 %, 1·0 c.c. of serum inactivates, during 30 min. of incubation, about 2·8 μg. of histamine diphosphate out of the initial 3·0 μg. As regards the placenta, 0·075 g. of the tissue inactivated between 70 and 92 % and 0·1 g. between 90 and 98 %. Calculated per g. of tissue, the amount of histamine inactivated by the placenta in 30 min. is between 28 and 40 μg. In other words, the histaminolytic action of the placenta is 10–15 times stronger than that of serum.

No significant difference could be detected in the histamine inactivation by placental extracts early in pregnancy and at full term. The total production of the histaminolytic agent depends, therefore, on the amount of placental tissue present at the moment and not on any qualitative difference of the tissue. In this respect it is of interest that the increase of the 'HI' in the maternal blood follows more closely the growth curve of the placenta than that of the foetus (Ballantyne, 1902).

The amniotic fluid and extracts of the umbilical cord had no histaminolytic action.

Observations on animals. Determinations of the 'HI' during pregnancy disclosed an important difference between animals and man. In contrast with human pregnancy, the 'HI' in animals remains unchanged throughout the period of gestation. Blood samples taken at the end of term in the dog (5), in the cat (5), in the rat (2) and in the mare (3) when incubated, even for as long as 2 hr., destroyed no histamine at all. In animals, which normally have a doubtful or a limited power to destroy histamine, such as the water buffalo (6), pig (2) and rabbit (8) the 'HI' of the serum remained, within the limits of the method, the same during the entire pregnancy, 10–30 %.

All attempts to demonstrate the presence of an histaminolytic agent in placental extracts of the water buffalo, pig, cow and dog gave uniformly negative results.

DISCUSSION

The unequal distribution of histaminase in the body was first detected by Best & McHenry (1930). Extracts of intestine, kidney and lungs had a powerful histaminolytic action, while those of muscle, blood, and many other organs, had either no action or only a limited action. It is a point of interest that a high histaminase and a high histamine content in the same organ are not incompatible. For example, the lungs, and especially the intestine, are rich in both, while the kidneys, independently of whether they are rich in hista-' minase, as in the dog, or poor, as in the rabbit, all have very little histamine. The coexistence of histamine and of the enzyme system destroying it suggests that, under normal conditions, they are not in effective contact with each other. The tissue histaminase is unable to act on the tissue histamine until the organ has been thoroughly macerated. To give only one example, a fresh piece of dog's intestine had 170 μg. histamine (as diphosphate) per g.; after incubation for 3 hr. the histamine content was unaltered; a similar incubation of the same intestine, after it had been ground up with silver sand, reduced the histamine content to about 65 μg.

Under normal conditions histaminase is a much less easily liberated constituent of the tissues than histamine. In fact, several conditions are known where histamine is liberated by the tissues into the general circulation. None, however, has been reported for histaminase. In this respect the human placenta is a unique organ which not only produces large amounts of histaminase but also generously liberates it into the maternal blood. To a small extent the histaminase diffuses also into the circulation of the foetus. Any speculation concerning the function of histaminase in the blood during pregnancy is as yet premature. It is tempting to ascribe to it a function of histamine detoxication. Histaminase is not, however, a specific enzyme and therefore it is quite possible that it has to be considered in connexion with some substances other than histamine. There is no evidence that the histamine content of the mother's blood, or the excretion of histamine in the urine, is different from that of non-pregnant individuals. Marcou reports a diminution in the blood histamine during labour. We cannot, however, confirm this observation. Furthermore, since the 'HI' is greatly increased throughout the entire last third of pregnancy, the histamine of the maternal blood might be expected to be lowered long before delivery; this is not, however, the case.

Of great interest is a comparison between the histaminolytic index and other auxiliary manifestations of pregnancy such as the production and excretion of oestrogenic and gonadotrophic hormones. On the one hand, there is a striking similarity between the 'HI' of pregnancy and the Aschheim-Zondek reaction.

No usual laboratory animal shows a detectable increase in the 'HI' during pregnancy and none, with a possible exception of the mare, gives the Aschheim-Zondek reaction. The latter is obtained in the chimpanzee. Unfortunately, our repeated attempts to obtain a blood sample from a pregnant chimpanzee, or even to get its placenta, only resulted in rather severe casualties (not to the chimpanzee). On the other hand, the curve of excretion of the chorionic gonadotrophin is quite unlike that of the increase in the 'HI'. The gonadotrophic hormone reaches a maximum on the 50–60th day of pregnancy and then falls during the next 20 days or so to a much lower level, which is then maintained during the rest of pregnancy. The Aschheim-Zondek reaction remains positive until from five to seven days after the birth of a full term child. In both respects the curve of the 'HI' of maternal blood is different. The increase of the 'HI' during pregnancy is progressive up to the end of term and its disappearance is much more rapid than that of the Aschheim-Zondek reaction.

The histaminolytic index follows a curve similar to that of the excretion of the oestrogenic principles during pregnancy. Both appear approximately in the 3rd month, both reach a maximum towards the end of pregnancy, and both rapidly fall to very low levels within about 48 hr. after labour.

The study of the 'HI' reaction in various pathological conditions of pregnancy is at present in progress.

Since the publication of our preliminary communication in 1941, several observers have contributed to the study of histaminolysis in pregnancy. The most important of these contributions has been made by Ahlmark (1944) who critically reviews the literature of the war period upon the subject and makes a detailed study of histaminolysis in human and animal pregnancy. Ahlmark states that most of the biological, gasometric and colorimetric methods used for the determination of the diamine oxidase reaction of blood were insufficiently controlled, that the observations were made under extremely varied conditions and that, therefore, the results, frequently contradictory, cannot be satisfactorily compared. All observers agree, however, that blood of pregnant women shows a conspicuous increase of histaminolysis.

On comparing our observations with those of Ahlmark we find that most of our results are confirmed with the exception of the following points:

(1) Ahlmark finds a weak histaminolytic action in the blood of man and of non-pregnant women. According to him the average inactivation of histamine by normal human plasma is $0.005 \mu g.$ of the base per c.c./hr. For the determination of this very small activity the plasma had to be incubated for 22 hr. The destruction of histamine during the entire period of incubation was, therefore, $0.11 \mu g./c.c.$ out of the initial concentration of $1.5 \mu g./c.c.$ of the base. We consider that the difference between $1.39 \mu g./c.c.$ and $1.5 \mu g./c.c.$ (about 9%) is too small for reliable estimation. Similar results were also obtained by us

when the serum was incubated for 24–36 hr. This very small activity does not seem to be due to histaminase since it is not abolished by raising the temperature of the serum-histamine mixture to above 80° C.

(2) Ahlmark's determinations of histaminolysis in animals do not always agree with ours. There is agreement as regards the male and the non-pregnant female of the rat and cat, in both of which no histaminolysis could be detected. In 15 normal guinea-pigs Ahlmark found an average histaminolytic activity of $0.16 \mu g$. base per c.c./hr. On recalculation of our results obtained in twenty-five animals, we find the same average, $0.15 \mu g$./c.c./hr. For rabbits, Ahlmark gives a figure of $0.23 \mu g$./c.c./hr. as compared with ours of $0.3 \mu g$./c.c./hr. The histaminolytic activity of the plasma of the cow, is given by Ahlmark as $1.5 \mu g$./c.c./hr., for the sheep $1.0–1.1 \mu g$. and for the pig $3 \mu g$./c.c./hr. Our estimations gave for these animals substantially lower figures ranging between 0.3 and $0.6 \mu g$./c.c./hr. of the base. In the horse, Ahlmark finds an inactivation of $0.2–0.3 \mu g$./c.c./hr. while we could find no destruction of histamine within the limits of the method.

Ahlmark finds that during pregnancy histaminolysis increases in most of the animals which he investigated. The increase is much smaller than in man and it is absent or doubtful in the rabbit and cow. According to our observations no significant change could be found in animals during pregnancy.

(3) In human pregnancy Ahlmark finds that the maximal histaminolysis is reached between the 6th and 7th months, after which it somewhat declines to rise again in the 9th month. We find a steady increase of the histaminolytic activity which reaches a maximum in the 9th month of pregnancy. Ahlmark states that between the 60th and 80th day of pregnancy it is possible to detect a rise in histaminolysis from day to day. We are unable to detect such a rise since the method of histamine determination is not sufficiently accurate and since changes in histaminolysis within 10% cannot be regarded as statistically significant. As a result of a large number of determinations made on the same patient, we consider that a significant increase of the 'HI' cannot be spotted with certainty at less than 7-day intervals.

Ahlmark finds an increase in histaminolysis, ranging from 0.0 to 58% within 2–3 hr. after parturition. According to our observations, serum samples collected a few hours before and a few hours after delivery have, within the limits of the method, the same 'HI'. The decline of the 'HI' begins about 8 hr. after delivery and then proceeds at the rapid rate of about 50% per day.

(4) Exceptionally high histaminolysis was found by Ahlmark in the placenta of the rat ($0.3–2.0$ mg./g./hr.) This observation, which we can entirely confirm, is of interest since during pregnancy rat's blood shows no histaminolytic activity or, according to Ahlmark only a very weak activity. The histaminolytic power of the placentae of the guinea-pig, rabbit and cat was found by Ahlmark to be much inferior to that of the rat. We investigated only the

placentae of the dog, water buffalo, cow, horse and pig; in all these we completely failed to detect any histaminolytic action. As regards the human placenta, the histaminolytic values were never as high as those reported by Ahlmark. Our figures, calculated per g. of placenta per hr., range between 20 and 30 μg. of the base as compared with 56–102 μg. found by Ahlmark. As stated in the text, no significant difference was found between the histaminolytic value of placental extracts, early and late in pregnancy.

SUMMARY

1. A rapid method for estimation of the histaminolytic action of blood is described, and a convenient measure of this action by an arbitrary 'histaminolytic index' ('HI') is suggested.

2. Within the limits of the method, no histaminolysis can be detected in the blood, plasma or serum of normal human subjects, or of patients suffering from various pathological conditions enumerated in the text.

3. Little or no histaminolysis is found in the blood of the usual laboratory and domestic animals.

4. A strong histaminolytic agent appears in the blood of women during pregnancy. Beginning from the third month of pregnancy, the histaminolytic action of the maternal blood gradually increases throughout the entire period of gestation reaching a maximum towards term.

5. The histaminolytic agent is found only in the plasma or serum. None is present in the red blood corpuscles.

6. After delivery, the histaminolytic action of the maternal blood rapidly declines, the 'HI' diminishing in 24 hr. to about a half, and in 48 hr. to less than a quarter of the maximum reached at the end of pregnancy.

7. Evidence is provided to show that the human placenta is the source of the histaminolytic agent in the maternal blood. The histaminolytic power of the placenta is, per g. of tissue, 10–15 times stronger than that of 9-months-pregnancy serum. Per g. of tissue, placentae of different age and weight do not differ greatly as regards their power to inactivate histamine.

8. With the method described in the text, no change in the histaminolytic action of the blood can be detected in animals during pregnancy and extracts of their placentae, with the exception of that of the rat, have no power to inactivate histamine.

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CORTICAL LIPIDS OF THE NORMAL AND DENERVATED SUPRARENAL GLAND UNDER CONDITIONS OF STRESS

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Acute experiments on anaesthetized animals have demonstrated a stimulating effect of intravenous infusions of adrenaline on the secretion of the suprarenal cortex (Vogt, 1944). The results led to the conclusion that every stimulation of the splanchnic nerves was accompanied by an increased activity of the cortical as well as of the medullary tissue. Splanchnic stimulation is known to be involved in the response of the organism to many types of stress. Further, changes in the lipid content of the cortex, probably associated with increased release of hormone, are known to occur under a variety of stress conditions. Changes were observed after exposure to abnormal environmental temperature (Ewert, 1935; Flexner & Grollman, 1939; Dosne & Dalton, 1941; Sayers, Sayers, Fry, White & Long, 1944; Levin, 1945); to low atmospheric pressure (Darrow & Sarason, 1944); after exercise (de Jongh & Rosenthal, 1933; Andersen, 1935; Knouff, Brown & Schneider, 1941); after insulin (Poll, 1925 *a. b*; Kahn & Münzer, 1927; Chamberlain, 1930); during fasting (Eger, 1942; Blumenthal & Loeb, 1942); after haemorrhage (Sayers, Sayers, Liang & Long, 1945); in vitamin B deficiency (Ralli & Graef, 1944); in shock (Popjak, 1944); during water intoxication (Gaunt, Cordsen & Liling, 1944) and in infectious diseases. Changes also occur after injections of adrenaline (Mazzeo, 1928; Selye, 1937; Long & Fry, 1945). The question thus arose whether the response of the adrenal cortex to such conditions is intensified, or possibly even produced, by the stimulation of the splanchnic nerves and the resulting secretion of adrenaline. The lipid content of the adrenal cortex was therefore examined in animals submitted to stress, and the reaction of normal animals compared with that of litter mates in which the adrenals had been denervated.

The results obtained by different authors on the changes in lipid content of the adrenal cortex under conditions of stress are so contradictory that the question had to be reinvestigated before the effect of denervation could be

examined. In the course of these experiments, the main reasons for the existing discrepancies in the literature soon became evident. They are, first, the considerable individual variations in both time course and intensity of the response to identical procedures. Secondly, the fact that, provided the stimulus is sufficiently prolonged, the reaction consists of two phases. The first phase is one of lipid depletion, the second is a restoration to normal or even an accumulation of lipids. This two-phase action has recently been stressed by Sayers *et al.* (1944), and is also seen after treatment with adrenaline: cholesterol loss was observed by Long & Fry (1945) after a single subcutaneous injection of adrenaline, whereas, if the injections are continued for days and weeks, accumulation of lipids ensues (Vogt, 1945).

The lipid content was assessed by microscopic examination of sections stained with one of the Sudan dyes. The results of this technique have been shown to be in good agreement with those obtained by histological or chemical cholesterol estimations (Sayers *et al.* 1944), and the method, though its results cannot be recorded in figures, occasionally gives information about loss of lipid which a chemical estimation of the cholesterol may not convey. The lipids disappear from the different layers in a characteristic order (Sarason, 1943), and patches of obvious depletion can be found in early stages, when the total loss, owing to the great variability of normal figures, may not be significant. All three methods have the disadvantage that a resting gland is not distinguishable from a gland which is in the process of beginning lipid accumulation. Hence, it was attempted so to time the exposure to stress that the glands would be examined during the phase of lipid loss, before restorative processes had become effective. Since this probably meant submitting the animals to stress for the duration of a few hours only, preliminary experiments were carried out in order to ascertain the effect of injections of adrenaline when given over a similar period.

METHODS

Choice of experimental animal. Both rats and cats were used. Rats have the great advantage that their adrenals have a small store of lipids, depletion of which is readily observed histologically. Complete denervation of their glands, however, involves injury to an artery running with the splanchnic nerves, and a small infarctation at the cranial surface of the adrenal is the invariable result, occasionally even atrophy of a whole gland. Though these lesions do not prohibit the observation of the lipid distribution, it was desirable to repeat the experiments on a species where denervation could be carried out without damage to the suprarenal blood supply. Cats were chosen, and litter mates reared in order to reduce the range of individual variation. The experiments, however, were a disappointment, as loss of lipids from the massive stores in their glands is far less conspicuous than in rats, and the lipid distribution varies considerably within the same litter.

Technical procedures. The effect of stress on the suprarenal gland was assessed by comparing the glands of the treated animals with those of an untreated litter mate of the same sex. When animals were exposed to stress after adrenal denervation, a normal litter mate was subjected to the same conditions of stress, and, whenever necessary, another normal litter mate was kept as un-treated control.

Adrenaline injections were given subcutaneously in doses of 0·25 mg./kg. at intervals of 2 hr. The animals were killed 2 hr. after the last injection.

The means used to produce 'stress' were low and high environmental temperature, haemorrhage and injections of insulin. Exposure to cold environment consisted of keeping the animals for periods of from 1 to 17 hr. at temperatures between +2 and +4° C. As food was allowed, no ill effects followed such treatment. For the experiments with high environmental temperature, the animals were placed for from 1 to 6 hr. in a box in which a temperature of 37–39° C. was maintained. During the longer lasting exposures, the rectal temperatures rose and weakness frequently ensued. Experiments on blood loss were done only on rats, 1–2% body weight of blood being withdrawn from the jugular vein under ether anaesthesia. The animals were killed a few hours later. Insulin was given by subcutaneous injection to animals which had been starved overnight. The animals were killed 1–7 hr. after the injection.

The suprarenal glands were fixed with Orth's fluid (Gatenby & Painter, 1937) to show the adrenaline, they were embedded in gelatin, cut by the freezing microtome and stained with haematoxylin and either Sudan III or Sudan IV. The glands to be compared were often cut side by side in the same block.

Denervation of the suprarenals was always performed bilaterally. The technique for rats is described elsewhere (Vogt, 1945). Evidence of the completeness and duration of the denervation will be given in the section on insulin. In cats, which were anaesthetized with ether after premedication with atropine, the operation was performed aseptically through a midline incision. The larger and lesser splanchnic nerves were divided, and the upper three lumbar sympathetic ganglia removed together with the sympathetic chains. Thus any fibres which might enter the adrenals without joining the splanchnic nerves were severed.

RESULTS

Adrenaline injections

The experiments showed that adrenaline, injected over a period of 8 hr., leads to a loss of sudanophilic material in the suprarenal cortex which is easily demonstrated with the histological method employed.

Four pairs of litter-mate rats, 34, 38, 49 and 84 days old, were given four injections of 0·25 mg. adrenaline per kg. body weight, and killed 2 hr. after the last dose. A conspicuous loss of lipid was found in the glands of all the injected rats, and ranged from moderate to severe depletion.

A similar experiment was carried out on cats, and the same dose and timing used. Here, the effect on a normal animal was compared with that on a cat submitted 21 days previously to suprarenal denervation. Both animals responded with a large loss in cortical lipids. Undoubtedly, therefore, non-toxic quantities of adrenaline, given over a period of 8 hr., caused changes which were similar to those reported as occurring under the influence of stress. If, however, two or three injections of adrenaline were given instead of four, the effect on cortical lipids was doubtful.

Exposure to low temperature

Rats. Exposure to low temperature for a short period (1 hr.) was not associated with a loss of sudanophilic material, but there was a depletion and sometimes even a complete disappearance of lipid from the zona fasciculata

after exposure for 14–17 hr. The depletion in lipids was no greater when food was withheld than when it was allowed, in spite of the fact that the 'stress' is more severe if rats are kept without food in a cold environment. All except a few preliminary experiments were, therefore, done on rats which had access to food.

The lipid loss occurred in the innervated as well as in the denervated suprarenals, as illustrated by the following experiment. One female and two male rats (aged 4–5 months) in which denervation of the adrenals had been performed 14- 17 days previously, were exposed, together with their normal litter mates, for 16 hr. to +4° C. Microscopic examination showed the same moderate lipid loss in the glands of all six rats. Not even in the normal animals, in which it might have been expected, was there visible depletion of the adrenaline stores.

Cats. Exposure to low temperature (+2° C.) for a period of 8–11 hr. produced a reduction in cortical lipids in only one of three pairs of litter-mate kittens, but when it occurred it was found both in the denervated and the innervated glands.

Exposure to high temperature

Rats. Normal adult rats (females of 110 g.), when kept at 39° C. for 1, 3 and 6 hr.. showed a lipid depletion which increased with the duration of exposure, all animals having developed large rises in rectal temperature (up to 42·5° C.) during these periods. Litter-mate (adult male) rats with normal and denervated adrenals were then compared. the operations having been performed 9 35 days before the exposure to 39° C. Depletion of lipid was found in many but not in all animals. The determining factor for the loss of lipid, however, was not the presence or absence of a nerve supply to the suprarenals, but the occurrence of a rise in body temperature. In those rats, in which the rectal temperature remained normal, the cortical lipids were not depleted, irrespective of whether their adrenals were normal or denervated. Neither did the integrity of the suprarenal nerves directly affect the course of the body temperature, as this was determined by the general behaviour of the rat. Those rats which slept quietly during the exposure to the warm environment had no fever, whereas animals which became excited and ran about developed rises in temperature ranging from 41·5 to 43·5° C. and would have died of heat stroke if not removed from the box. The normal controls were more inclined to violent exercise and concomitant rise in temperature, so that fever and loss of lipids occurred in seven out of eight normal and in six out of eleven operated rats, but any degree of lipid depletion could be obtained in the operated animals, provided a sufficient rise in body temperature had been induced.

The adrenaline stores were not appreciably reduced in any of the innervated glands, not even in those from animals in which a loss of lipids had occurred.

Cats. Only an inconspicuous reduction of the lipid content of the adrenals was obtained by an exposure of cats to warm environment which was sufficient to produce body temperatures of 41·2–44·0° C., but the small responses observed were unaffected by the excision of the nerves to the suprarenals.

Haemorrhage

Sayers *et al.* (1945) observed a 50% fall in cortical cholesterol in rats which had been bled 3 hr. previously. The following is a description of a few experiments on rats in which the effect of adrenal denervation on this phenomenon was investigated. In a first pair of litter mates the quantity of blood withdrawn amounted to 1% of the body weight, and the rats were killed 2 hr. after the end of the haemorrhage. Lipid depletion was doubtful in both rats. More severe haemorrhage was, therefore, produced in the next experiments. Withdrawal of 2 c.c. of blood/100 g. body weight was easily carried out in normal rats, but no more than 1·4–1·6 c.c./100 g. could be removed in the splanchnotomized animals. Yet, this led to more serious effects than the larger haemorrhage in the normal animals. The rats were killed 4–5 hr. after the end of bleeding. Loss of cortical lipids was found in all the animals, and was more severe in the operated rats than in the controls. Depletion of lipids obviously ran parallel to the severity of clinical signs, and the denervation of the suprarenals apparently affected the results only in so far as it rendered the rats more susceptible to shock from haemorrhage. In no instance was there a visible loss of adrenaline from the innervated medulla.

Insulin injections

Normal rats. When the same dose of insulin is injected into a group of starving rats and the animals are examined a few hours later, the clinical signs, the blood-sugar concentration and the appearance of the suprarenal cortex vary considerably. Age and sex influence the response. Even among litter mates of one sex variations occur, but, by comparing rats of one litter, it is possible to establish certain rules governing the response to different doses. Small quantities of insulin, if at all effective, cause lipid depletion in the cortex and, with rising doses, the effect increases up to a certain limit. If, however, the dose exceeds that limit, pictures of depletion of lipids alternate with those of normal or even excessive stores of lipid. Thus insulin, according to dosage, may cause depletion or storage of cortical lipids, just as administration of adrenaline may produce either of these two effects.

The minimal dose required to cause loss of lipids from the suprarenal cortex of rats, examined 2½–6½ hr. after a single injection. was 0·12 i.u. insulin/100 g. body weight. At this level, depletion of the adrenaline stores from the medulla was not yet visible, but it became obvious at 0·24 i.u. and was very conspicuous with all doses above this level. Clinical signs rarely appeared before 0·4–0·8 i.u. had been given. If the effect of increasing amounts of insulin on

the cortical lipids was tested on different rats with doses ranging from 0·3 to 15·0 i.u./100 g., a normal content of sudanophilic material was found in some and a reduced content in other animals. Whereas litter mates treated with different doses tended to have identical reactions, there was no correlation of the response with either the duration of the experiment or with the dose. It was suspected that those rats which showed 'normal' amounts of lipids after the larger doses of insulin were in the recovery phase of the response, and that variations in the time course of disappearance and formation of lipids in the cells might explain the erratic results.

This explanation was put to the test in an experiment in which the high single doses, the effects of which cannot be followed up for a sufficient period of time as they kill the rats, were replaced by repeated injections of tolerated doses. Four litters, each of four adult males, were used. One rat of each litter was kept as control and not given insulin, a second rat had one injection of 0·3 i.u./100 g. and was killed after 3 hr., and the last two animals were injected twice with 0·3 i.u./100 g. at an interval of 3 hr. and were killed 4 hr. after the second injection. In three of the litters, the single injection caused severe lipid depletion. The glands of their litter mates given a second dose contained normal or only slightly reduced quantities of lipid. Obviously the second injection had produced partial or complete recovery from the original loss. Not so in the fourth litter. Here, very little depletion was caused by the first injection, and severe loss followed the second dose. Thus the result of the prolonged treatment varied with the susceptibility of the litter, animals of a susceptible litter being already in a phase of recovery when rats from a less sensitive litter were still losing lipids.

Rats with denervated adrenals. For the comparison of the effect of insulin on innervated and denervated adrenals, single injections of 0·3–0·5 i.u. insulin/ 100 g. body weight were given and the experiments interrupted after 3–4½ hr. According to the information reported in the last section, these conditions will cause depletion of lipids in the cortex of at least a large proportion of normal rats. Males were used predominantly, as the loss of lipids is less easily elicited in females. The denervation was performed 15–84 days before the insulin was injected. None of the operated injected rats showed any sign of adrenaline loss in the chromated sections, whereas a very large depletion of the adrenaline stores was visible in all the injected normal rats. This proves that, at least from a physiological point of view, the denervation was complete and remained so up to a period of 84 days. Atrophy of one denervated suprarenal, with hypertrophy of the remaining gland, occurred in a few instances. Some operated rats were not injected. Their glands were stained, and quantity and distribution of lipids were found normal in all specimens.

The results are summarized in Table 1. The rats in each single experiment are litter mates. The clinical signs in all but two operated rats were more

severe than in the normal rats, which often had no signs at all. Blood-sugar estimations showed severe hypoglycaemia in the operated rats only.

TABLE 1. Effect of insulin on rats with normal and denervated adrenals

Exp. no.	Age (months)	Time since dener-vation (days)	Dose (i.u./100 g.)	Blood sugar (mg./100 c.c.) Normal	Operated	Signs Normal	Operated	Lipid loss Normal	Operated
				Male rats					
1	2½	15	0·3	122	65	None	Paralysis	+ +	+ +
			0·3		59		Paralysis		0
2	3	15	0·3	78	52	None	Paralysis	+ +	+ +
			0·3		46		Paralysis		0
3	5	24	0·3			None	Paresis	+ +	0
4	5½	16	0·3			None	Paresis	+ +	0
			0·3				Paresis		0
5	3	21	0·4			Paresis	Paralysis	+ + +	+ +
6	3	21	0·4			None	Paresis	+ -	+ +
7	3½	29	0·4			Paresis	Paresis	+ +	+ + +
8	3½	29	0·4			Weakness	Greater weakness	+ +	+ + +
9	3½	30	0·4			None	Paresis	+ ?	+ ?
10	4	33	0·4			None	Paralysis	+ +	+ +
		—					None*		0*
11	5	18	0·5			Coma	Coma	0	0
		—					None*		0*
				Female rats					
12	8	84	0·5			None	Paralysis	+	+ +
			0·5			None	None	+ + +	+ +
		—					None*		0*

Rats marked * had no insulin.
0 = no lipid loss.   + = some lipid loss.   + + = large lipid loss.   + + + = very large lipid loss.

Comparison of the adrenal cortex in denervated and in normal glands shows that, in ten of the twelve litters, lipid loss was obvious in the normal adrenal. In the denervated glands from these ten litters (fourteen rats), loss of lipids was absent in five and present in nine animals, and in two of these was more conspicuous than in the normal glands. Hence, insulin is able to cause loss of lipid in denervated adrenals, and must do so by means other than by causing secretion of adrenaline. Since, however, depletion of lipids occurs less frequently when the glands are denervated, adrenaline must be regarded as a contributing factor to the loss of cortical lipid.

The experiments in which the blood glucose was tested allow yet another conclusion. The adrenaline secretion caused by the insulin in the normal rats was sufficient to prevent a severe fall in blood sugar and the occurrence of clinical signs. As there was, however, a conspicuous loss of cortical lipids in these animals, it follows that this loss is not a result of the hypoglycaemia.

Cats. Six pairs of litter-mate kittens were used, and denervations performed on one animal of each pair. Cats were found to be 5–10 times more sensitive to insulin than rats. Doses of insulin ranging from those which do not produce any signs (0·2 i.u./kg.) up to levels which cause unconsciousness after 1½ hr.

(1·6 i.u./kg.) were tried. With the larger doses, blood-glucose levels fell to about 40 mg./100 c.c. blood. As was to be expected, the clinical signs were much more severe in the operated cats, and no loss of adrenaline was detectable histologically in the medulla of their adrenals. Thus denervation can be presumed to have been complete.

None of these doses, nor any variation in the duration of the experiments (1½–4½ hr.), produced a depletion in cortical lipid which could be established with certainty, in spite of the fact that, in the normal controls, one-half to two-thirds of the medullary cells had lost all their adrenaline.

These results are surprising in so far as adrenaline given subcutaneously was found to cause depletion of cortical lipids in cats. The explanation may be that the adrenaline secreted by the suprarenals in response to insulin administration was insufficient in quantity. Assuming the adrenaline content of the two glands of a cat to be 0·4 mg. (Elmes & Jefferson, 1942), of which 0·3 mg. are released through the action of insulin, the dose of 4 × 0·25 mg. adrenaline injected subcutaneously at 2-hourly intervals, and followed by lipid depletion, is higher, and may account for the greater effect. The importance of the dose is stressed by the observation that two and three injections of 0·25 mg. adrenaline were found to be far less effective than four injections. On the other hand, the discrepancy may be due to the fact that the time course of adrenaline secretion produced by insulin differs fundamentally from that of absorption of adrenaline after subcutaneous injections.

DISCUSSION

The conditions of stress employed in this investigation fall into two groups. In the first group, which comprises changes in environmental temperature and blood loss, the stress produced loss of lipid in the suprarenal cortex without visible depletion of the adrenaline stores in the medulla. Nevertheless, adrenaline must have been released under those conditions. Its release on exposure to low and high temperature can be inferred from physiological evidence and from the anatomical work of Staemmler (1933) who detected a loss of adrenaline in sections of the adrenals taken from animals kept in a cold or warm environment. Staemmler's conditions were possibly more severe than those used in the present investigation, and the difference in the release of adrenaline may be one of degree only. In the experiments on haemorrhage, the loss of blood was poorly withstood after suprarenal denervation, and this is to a large extent a result of the lack of adrenaline secretion. In all these experiments loss of cortical lipids was found to be independent of adrenal innervation. Hence, these conditions of stress did not require release of adrenaline to bring about their action on the suprarenal cortex. They probably caused metabolic changes in the organism which led to a secretion of corticotrophic hormone from the anterior lobe of the pituitary gland which resulted in depletion of cortical lipids (Sayers, Sayers, White & Long, 1943).

In the second group, in which stress was produced by insulin, there was not only depletion of lipids in the cortex, but also conspicuous loss of adrenaline from the medulla. This loss of adrenaline occurred in spite of the fact that the doses of insulin were often too small to produce clinical signs. Cannon, McIver & Bliss (1924) were the first to show that insulin causes secretion of adrenaline, and the resulting depletion of the stores in the medulla was shown histologically by Poll (1925 a, b). Cannon *et al.* (1924) demonstrated the physiological importance of this secretion by showing that the clinical signs which follow injections of insulin are much more severe in sympathectomized than in normal animals. This fact was confirmed in the present experiments on the rats and cats with denervated adrenals.

Structural changes in the cortex after high doses of insulin were first seen by Poll (1925 a, b). Kahn & Münzer (1927) identified these changes as loss of lipid and attempted to decide whether or not they were dependent on the integrity of the splanchnic nerves. In two out of three rabbits they found no lipid loss if one suprarenal had been removed and the other denervated. Lipid loss usually persisted, however, in the denervated gland after unilateral section of the splanchnics, much to the surprise of the authors, who attributed the result to crossed innervation. We know to-day that unilateral splanchnotomy cannot solve the problem since it does not prevent adrenaline from being released into the circulation and thus affecting the lipid stores of the cortex.

The results of the foregoing experiments on animals with bilateral denervation have shown that the adrenaline secretion contributes to the loss of lipids caused by insulin, as this loss failed to occur in some of the rats with denervated glands. Since, however, it did occur in the remaining rats, we must assume that insulin can cause depletion of cortical lipids in yet another way, and presumably by the release of corticotrophic hormone from the anterior pituitary, as this is the mechanism by which depletion of suprarenal lipid stores is usually produced. This effect of insulin on the pituitary may be through a direct action or may be caused indirectly by the metabolic disturbances which occur after insulin. If these disturbances were mainly responsible, the stimulation of the anterior lobe by insulin would be very strong after suprarenal denervation which prevents the hypoglycaemia and clinical signs from being counteracted by the secretion of adrenaline, but it might be negligible in animals with *innervated* suprarenals, if doses of insulin causing a mild fall in blood sugar were not exceeded. With such doses, therefore, the disappearance of cortical lipids and thus presumably the increase in cortical secretion might be entirely due to the release of adrenaline. If, however, insulin did not act only through the intermediary of metabolic disturbances, but stimulated the anterior lobe directly, the adrenaline secretion would have to be regarded as no more than a contributing factor in the causation of lipid loss. Whatever the mode of action of insulin on the pituitary

may be, the results obtained at dose levels at which clinical signs are absent leave no doubt that both adrenal cortex and medulla are involved in the physiological response to insulin long before manifestations of hypoglycaemia become evident.

The role played by splanchnic stimulation in the loss of cortical lipids under conditions of stress can be summarized as follows: lipid depletion as a result of low or high environmental temperature and haemorrhage does not require the action of the splanchnic nerves, but the depletion caused by insulin is to some extent a result of splanchnic stimulation. This may be due to the larger size and speed of adrenaline secretion after insulin.

SUMMARY

1. Repeated injections of adrenaline, given to rats or cats over a period of 8 hr., cause a conspicuous loss of sudanophilic material in the suprarenal cortex.

2. Denervation of the adrenals does not prevent the depletion of lipids observed in rats under the following conditions of stress: (a) exposure to low temperature ($+2$ to $+4^\circ$ C.) for 16 hr., (b) exposure to high temperature (39° C.) for 2–6 hr. (provided a rise in body temperature is produced), and (c) loss of blood amounting to 2 % of the body weight.

3. In rats, lipid depletion in the adrenals is usually observed after injection, per 100 g. body weight, of 0·12 i.u. insulin or more, and adrenaline loss after 0·24 i.u. or more. Clinical signs occur after 0·6 i.u. or more. If the dose is raised further, or if the injection of a smaller dose is repeated, lipid depletion may be replaced by lipid storage. Adrenal denervation inhibited the loss of lipid in some but not in all animals. Hence, secretion of adrenaline contributes to the loss of cortical lipids seen after insulin injection, but is not indispensable for it. The difference between the effect of adrenal denervation on the response to insulin on the one hand, and on the reaction to abnormal environmental temperatures or haemorrhage on the other, may be the result of the greater magnitude and speed of the adrenaline secretion elicited by insulin.

4. In cats, with doses of insulin ranging from 0·2 i.u./kg. to 1·6 i.u./kg., it was not possible to obtain a definite loss of cortical lipids. Since the injections of insulin produced all degrees of depletion of adrenaline, up to very serious ones, it appears that release of the greater part of the adrenaline stored in both adrenals of a cat can take place without causing conspicuous changes in the very large lipid stores of the cortex.

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THE EFFECT OF CALCIUM IONS ON THE RESPIRATION OF KIDNEY SLICES OF NEWBORN AND MATURE RATS

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The respiration of kidney slices taken from mature and newborn animals was studied by Cutting & McCance (1946 *a*, *b*). Tissues of rats, cats, pigs and man were investigated. Among the differences found were the following: (*a*) During the 2 hr. period of shaking in the Barcroft flasks the mature tissues lost a larger proportion of their nitrogen to the surrounding medium than did the newborn tissues. In adult rats these losses ranged from 35 to 50 % of the nitrogen in the fresh tissue, and in newborn rats the corresponding losses were about 17 %. (*b*) The O_2 uptake (particularly in rats) fell off much less rapidly in tissues from newborn animals than it did in tissues from adult animals. (*c*) When the pH of the adult tissues was reduced from 7·4 to 6·8 (1) the initial O_2 uptake was unaltered (rats) or slightly depressed, (2) the O_2 uptake was better maintained, (3) the loss of nitrogen was reduced. The newborn tissues were relatively unaffected by this change of pH, but, in rats, the average O_2 uptake was slightly reduced at the lower pH both in the first and second hour. The loss of nitrogen was not reduced.

In all this work the fluid bathing the cells was ' Ringer-phosphate ' at pH 7·4. No Ca was added to this medium because all of it appears to be precipitated at this pH. The following considerations led us to formulate the hypothesis that some of the effects of changed pH observed on adult tissues might be due to a Ca deficiency: (*a*) Although it is not possible to prepare a Ringer-phosphate solution at pH 7·4 containing more than traces of Ca ions in solution, it is possible to do so at pH 6·8. Consequently, apart from Ca ions in the medium, any Ca ions in the tissue fluids bathing the cells are likely to be precipitated at pH 7·4 but not at pH 6·8. Although very small in amount, these residual tissue ions may be very potent. (*b*) Calcium ions are known to affect the permeability of membranes, and the presence of such ions in solution might possibly prevent the disintegration of the cells and some of the loss of nitrogen.

A number of workers have studied the effect of Ca ions upon the metabolism of mammalian tissues in vitro. Their results have not been wholly concordant but they seem to have established the fact that minced (or homogenized) tissues and tissue slices must be considered separately. Thus, Thunberg (1909 *a*, *b*)

originally showed that Ca ions inhibited the respiration of minced muscle tissue in Ringer-phosphate and this has been confirmed (Greville 1936, 1938). Elliott & Elliott (1939) found that Ca ions inhibited the O_2 uptake of minced liver, and Holck (1934) and Krebs & Eggleston (1938) that Ca ions had a similar effect upon minced renal cortex. Holck (1934) also noted that, although Ca inhibited the initial uptake of O_2, it tended to prevent the O_2 consumption falling off so rapidly. Hence. at the end of the experiments, the tissues incubated with Ca might be taking up O_2 more rapidly than those incubated without Ca. Elliott & Libet (1942) made the observation that Ca ions depressed the O_2 uptake of homogenized rat brain during the first 30 min. of observation but prevented the O_2 uptake from falling off so much during the second hour.

In contradistinction to this, Ca ions have not been found to depress the O_2 uptake of tissue slices. Leibowitz (1930) found that Ca ions promoted the O_2 uptake of cat kidney slices in Ringer-bicarbonate solution at pH 7·3-7·4, but had little or no effect upon the slices taken from the kidneys of some other animals. Lasnitzki & Rosenthal (1929) claimed that the anaerobic lactic acid production of tumour slices in bicarbonate-glucose-Ringer's solution was increased by the presence of Ca ions. Kisch (1934), working with a Ringer-bicarbonate solution at pH 7·4, and without the addition of oxidizable substrates, found that Ca ions improved or did not alter the O_2 uptake of rat kidney slices. When oxidizable substrates such as lactate were added to the Ringer-bicarbonate, Ca ions depressed the uptake of O_2. He further found that, with or without substrates, Ca ions improved the O_2 uptake of liver slices and depressed the O_2 uptake of strips of diaphragm. Krebs & Eggleston (1938) reported that Ca ions had no effect upon the O_2 uptake of slices of liver or of renal cortex respiring in 0·1 M-phosphate buffer.

If it is possible to draw any conclusions from the somewhat contradictory results of previous workers, it would appear that Ca ions in physiological amounts depress the O_2 uptake of all minced or homogenized tissues and improve slightly or do not alter the O_2 uptake of slices of mammalian liver or kidney. Krebs & Eggleston (1938) and Greville (1938) explained these results by suggesting that when Ca was added to homogenized tissues the ions had access to intracellular enzymes, from which they were normally debarred, and inhibited them. One might amplify this suggestion by supposing that, when Ca was added to the medium in which the O_2 uptake of tissue slices was being measured, the ions remained outside the cells and exerted their physiological role on the membranes.

Lasnitzki (1933) appears to have been the only worker to compare the effect of Ca ions on adult and on embryonic tissues, and rather unusual times were selected over which to make measurements. Using a Ringer-bicarbonate medium this author found that Ca ions increased considerably the O_2 uptake of slices of adult rat kidneys between 90 and 120 min. after the experiments

had been set up. On the other hand, Ca ions had no effect upon the O_2 uptake of slices taken from embryonic kidneys. It must have been difficult to cut slices of embryonic rat kidneys removed in the last third of pregnancy. Unfortunately, this is a very short paper, and it contains very little information about technique and only fragmentary data. The author drew the conclusion that 'die Kationenabhängigkeit der Atmung…eine Eigenschaft der Gewebe ist, die im Laufe ihrer Entwicklung erworben wird und sich allmählich immer stärker herausbildet'.

METHOD

Kidney slices from newborn and mature rats were prepared as described by Cutting & McCance (1946a), and their respiration was measured in the same Barcroft apparatus. Slices were suspended in the following solutions: (1) Ringer-phosphate at pH 6·8—without calcium. To 103 vol. of 0·9 % NaCl were added 4 vol. of 1·15 % KCl, 1 vol. of 3·82 % $MgSO_4$. $7H_2O$, 1 vol. of 2·11 % KH_2PO_4 and 21 vol. of phosphate buffer pH 6 8. (2) Ringer-phosphate at pH 6·8—with calcium. 2·64 c.c. of solution (1) were measured into the Barcroft flasks and 0·06 c.c. of neutralized 1·21 % $CaCl_2$ (standardized with $AgNO_3$ to be =0·11 M) were added. (3) Ringer-bicarbonate at pH 7·4—without calcium (Krebs & Henseleit, 1932). To 103 vol. of 0·9% NaCl were added 4 vol. of 1·15% KCl, 1 vol. of 3·82% $MgSO_4$, $7H_2O$, 1 vol. of 2·11% KH_2PO_4, and 21 vol. of 1 3% $NaHCO_3$. (4) Ringer-bicarbonate at pH 7·4—with calcium. Like solution (3), except that only 100 vol. 0·9% NaCl were taken and 3 vol. of 1·21% $CaCl_2$ (standardized with $AgNO_3$ to be =0 11 M) added.

2 7 c.c. of the Ringer's solutions (1) and (2) were placed in the outer compartment of the Barcroft flasks and 0·3 c.c. of NaOH in the central cup. 3 c.c. of Ringer's solutions (3) and (4) were used in the outer compartment. The Barcroft vessels containing Ringer-phosphate were always evacuated twice, and refilled with O_2 on each occasion, before the manometers were set up on the frame outside the water bath. Those containing Ringer-bicarbonate solutions were either not evacuated at all or evacuated twice and refilled with a mixture of 95% O_2 and 5% CO_2.

RESULTS

Tables 1 and 2 show the results of experiments in Ringer-phosphate at pH 6·8 with and without Ca. The O_2 uptakes over the first and second hours and the percentage losses of nitrogen of the adult kidney slices are given in Table 1, and the same data about the newborn kidney slices in Table 2. The results without Ca agree with those of previous experiments in showing that kidney slices from the newborn rat had a smaller initial O_2 uptake than slices from the adult, but that the rate of uptake was better maintained. They also show, as before, that the slices from the newborn lost much less of their nitrogen. Each line of Table 1 gives the results of one experiment carried out on the tissues of a single rat, and each line of Table 2 the results of an experiment on a single litter. It will be observed from Table 1 that over the first hour the addition of Ca slightly reduced the O_2 uptake, but the difference between the averages was not significant. During the second hour, however, the O_2 uptake was better maintained in the presence of Ca ions and the O_2 uptake was significantly greater during this period. It will be recalled that Holck (1934) made similar observations on minced renal tissues. It will be noted also that Ca ions

TABLE 1. The effect of calcium ions on the O_2 uptake by, and on the losses of nitrogen from, kidney slices of adult rats respiring in Ringer-phosphate at pH 6·8

	Ringer-phosphate at pH 6·8			Ringer-phosphate + Ca at pH 6·8		
	O_2 uptake c.mm./min./mg. N during		loss of N by slices	O_2 uptake c.mm./min./mg. N during		loss of N by slices
Rat no.	1st 60 min.	2nd 60 min.	%	1st 60 min.	2nd 60 min.	%
1	103·0	72·5	35·0	96·0	88·0	22·0
2	91·5	76·5	28·6	76·5	78·0	23·3
3	107·0	66·5	34·0	97·0	94·0	35·0
4	126·0	59·5	27·0	110·0	88·0	24·0
5	86·0	76·0	36·0	104·0	91·0	25·0
6	93·0	55·5	46·5	96·0	63·0	27·8
7	140·0	113·0	26·6	147·0	114·0	18·0
8	141·0	96·0	30·5	153·0	125·0	23·6
9	125·0	100·0	25·2	129·0	104·0	22·6
10	143·0	104·0	31·4	135·0	115·0	19·8
11	146·0	127·0	24·9	119·0	102·0	21·0
12	122·0	95·5	29·8	142·0	111·0	25·4
13	115·0	93·0	27·8	131·0	113·0	22·5
14	142·0	109·0	28·0	122·0	123·0	20·4
Average	120·0	88·8	30·8	118·4	100·6	23·6

The addition of Ca caused a significant increase in the O_2 uptake during the second hour ($t = 3·186$ when $P < 0·01$), and a significant decrease in the loss of N over the two hours ($t = 5·03$ when $P < 0·01$).

TABLE 2. The effect of calcium ions on the O_2 uptake by, and on the losses of nitrogen from, kidney slices of newborn rats respiring in Ringer-phosphate at pH 6·8

	Ringer-phosphate at pH 6·8			Ringer-phosphate + Ca at pH 6·8		
	O_2 uptake c.mm./min./mg. N during		loss of N by slices	O_2 uptake c.mm./min./mg. N during		loss of N by slices
Litter no.	1st 60 min.	2nd 60 min.	%	1st 60 min.	2nd 60 min.	%
1	94·0	82·5	21·1	85·0	85·0	24·1
2	89·0	93·5	18·0	62·0	68·0	23·5
3	107·0	119·0	19·6	111·0	107·0	14·8
4	129·0	107·0	13·8	117·0	90·5	15·7
5	110·0	94·5	14·5	110·0	95·0	15·5
6	101·0	86·5	16·9	83·0	79·0	12·4
7	108·0	92·0	16·5	88·0	77·5	15·4
8	109·0	106·0	10·0	89·0	81·0	14·7
9	99·0	107·0	14·7	106·0	106·0	13·5
10	112·0	101·0	17·3	114·0	112·0	18·0
11	101·0	106·0	16·8	108·0	104·0	14·8
12	95·5	88·0	19·8	100·0	90·5	18·3
Average	104·5	98·7	16·5	97·7	91·3	16·7

When there was no calcium in the medium the O_2 uptake of the adult tissues in the first hour was significantly greater than that of the newborn tissues ($t = 2·85$ when $P = 0·015$). In the second hour the difference between the adult and the newborn O_2 uptakes was not significant ($t = 1·7$ when $P = 0·1$). There was clearly a significant difference between the adult and the newborn tissues so far as the losses of nitrogen were concerned.

considerably reduced the percentage loss of nitrogen. All these effects may also be produced by lowering the pH from 7·4 to 6·8 (Cutting & McCance, 1946a). Table 2 shows that the addition of Ca ions to slices of kidneys from newborn rats slightly reduced the average O_2 uptake during the first and also during the second hour and that it did not reduce the loss of nitrogen. These differences are not statistically significant in either the first or the second hour, but they are similar in amount and direction to the results obtained when the pH was lowered from 7·4 to 6·8 (Cutting & McCance, 1946a).

Ca ions have also been shown to reduce the amount of nitrogen lost by adult tissues respiring in Ringer-bicarbonate solution at pH 7·1 under an atmosphere of 95 % O_2, 5 % CO_2, and in a similar solution at pH 7·4 under air. The results are given in Table 3. Each line shows the results of comparisons made with the tissues of a single animal.

TABLE 3. The effect of calcium ions on the loss of nitrogen by kidney slices of adult rats respiring in Ringer-bicarbonate solutions

Percentage loss of N in air at pH 7·4		Percentage loss of N in 95 % O_2 +5 % CO_2 at pH 7·1	
Without Ca	With Ca	Without Ca	With Ca
58·0	47·0	51·0	38·0
51·5	44·0	41·0	37·6
58·0	50·0	41·0	32·0
54·0	38·0	23·4	21·6
37·6	14·0	32·5	27·8
Av. 51·8	38·6	37·7	31·4

DISCUSSION

The effects of Ca ions, which have just been described, on the uptake of O_2 by adult kidney slices are on the whole in keeping with those of previous authors (Leibowitz, 1930; Lasnitzki, 1933; Kisch, 1934). If differences in technique and in the timing of observations are taken into account there is little doubt that all the workers have had the same basic phenomenon under observation. The action of Ca ions in reducing the loss of nitrogen has not been reported by previous workers, and it seems reasonable to associate the maintenance of the O_2 consumption with this reduced loss of nitrogen from the tissue cells. There are, however, points of greater interest in the results now reported, for Ca ions do not affect slices from the kidneys of newborn animals in the same way as they do those from the kidneys of adult animals. In particular, the O_2 consumption is not raised during the second hour, nor is the loss of nitrogen reduced. The present results confirm and extend those of Lasnitzki (1933), who must be considered to have been the first to detect that Ca ions might not have the same effects at all ages. If the ions are visualized as exerting their effects by combining with free phosphoric acid radicles in the cephalin molecules of the lipid-protein membranes (Hurst, 1946), then it would appear that these membranes must be quite differently constituted at birth and at maturity.

This may be a really fundamental feature of development, and one which is probably open to experimental attack. Secondly, this work was taken up on the assumption that the effects of lowering the pH, which had previously been observed (Cutting & McCance, 1946 a), might have been due to this change in pH retaining Ca ions in solution. The present results support this assumption, for the addition of Ca ions has been shown to reproduce all the effects of lowering the pH from 7·4 to 6·8. Even if this theory should not turn out to be correct it is highly probable that the effect of lowering the pH and the addition of Ca ions operate on the same cellular structures.

SUMMARY

1. The addition of Ca ions in physiological amounts to slices of adult rats' kidneys respiring in 'Ringer-phosphate' solution at pH 6·8 (a) does not alter the O_2 uptake in the initial stages, (b) maintains the O_2 consumption at a higher rate during the second hour of observation, (c) reduces the losses of nitrogen from the slices.

2. The addition of Ca ions under the same conditions to slices of newborn rats' kidneys (a) slightly reduces the O_2 consumption during the first and second hours of observation, (b) does not reduce the losses of nitrogen.

3. These effects are similar to those produced by lowering the pH of the medium bathing the slices from 7·4 to 6·8.

4. The addition of Ca ions to slices of adult kidneys respiring in 'Ringer-bicarbonate' solutions reduces the loss of nitrogen from the slices.

5. On the basis of these results it is submitted that the cellular membranes at birth may not have the same lipid-protein structure as they have at maturity.

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THE EFFECT OF ELECTROLYTE DEFICIENCY ON THE RATE OF CONDUCTION IN A SINGLE NERVE FIBRE

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It has been shown by Hodgkin (1939) that a change of resistance on the outside of a nerve fibre is followed by a change in conduction velocity. This effect was obtained (i) by altering the volume of saline surrounding the fibre, (ii) by placing a metal shunt in parallel with it. In the present paper, similar changes of conduction velocity are described following the replacement of electrolytes by sucrose.

METHOD

Isolated nerve fibres from the meropodite of the walking limb of *Carcinus maenas* were used. The technique of dissection and of immersing a portion of the fibre in saline or paraffin oil was similar to that described by Hodgkin (1938, 1939). The arrangement of stimulating and recording electrodes is shown in Fig. 1. Brief thyratron discharges were applied through platinum wires, and the action potential was recorded at two places (*CD* and *EF*, Fig. 1) just before it entered, and after it emerged from, the tested portion of the fibre. The solution consisted of a mixture of volumes of sea water and 1·0 molal sucrose (1 g.mol. sucrose to 1000 g. H₂O) which is approximately isotonic with sea water. The loop of fibre between *D* and *E* was held taut at its centre by a fine glass hook. The upper portions of the fibre, surrounded by a film of sea water, remained in paraffin oil throughout the experiment. The fibre was lowered into the required solution until the interface touched the electrodes *D* and *E*. It was then lifted slightly until interface and electrodes *D* and *E* were separated by about 0·5 mm., and the action potential was recorded in this position. The whole fibre was

Fig. 1. *N, Carcinus* nerve fibre. *A, B*, stimulating electrodes. *C, D* and *E, F*, two pairs of recording electrodes. The fibre was held at the ends by screw-controlled pairs of forceps and was depressed at its centre by a glass hook (not shown).

then lifted into paraffin oil and the action potential recorded again. Attention was paid mainly to the comparison of velocities in 'oil' and 'solution', with any given saline/sucrose mixture, as this comparison should be unaffected by membrane changes induced by electrolyte deficiency (see, however, Discussion). As the distances between *C* and *D*, and *E* and *F* were only

about 0·5 mm., quick diphasic spikes were recorded which crossed the base-line at a high rate (cf. Schmitt, 1939). The distance between the two points of intersection with the base-line could be measured very accurately, and conduction times could thus be determined to within 10 μsec.

To compare the conduction velocities in 'oil' and 'solution', the time for conduction between the mid-points of *CD* and *EF* respectively and the interface, 0·75 mm. away, must be deducted from the total conduction time. For example, if the total distance between the mid-points of *CD* and *EF* was 15·5 mm., and the conduction time of the 'sea-water axon' 2·52 msec. with the loop in saline, but 4 msec. with the loop in oil, a deduction of 0·4 msec. (conduction in oil over 1·5 mm. of fibre) must be made from both values to obtain an accurate ratio of velocities in oil and saline. When, for example, a 50 % sucrose/sea-water mixture is used, the deduction becomes somewhat larger (0·53 msec. in the case of Table 1 below), because the velocity of the impulse between the interface and *D* (or *E*) is rather less. In Table 1, both original and corrected ratios of impulse velocities are shown.

The electric resistance of saline/sucrose mixtures was determined in a conductivity cell with alternating current of 300 and 600 cyc./sec. The relative resistivities of the solutions are shown in the appropriate tables below.

RESULTS

The results are illustrated in Fig. 2 and Tables 1 and 2. In Fig. 2 the following points require attention: (i) If one follows the upper horizontal set of records
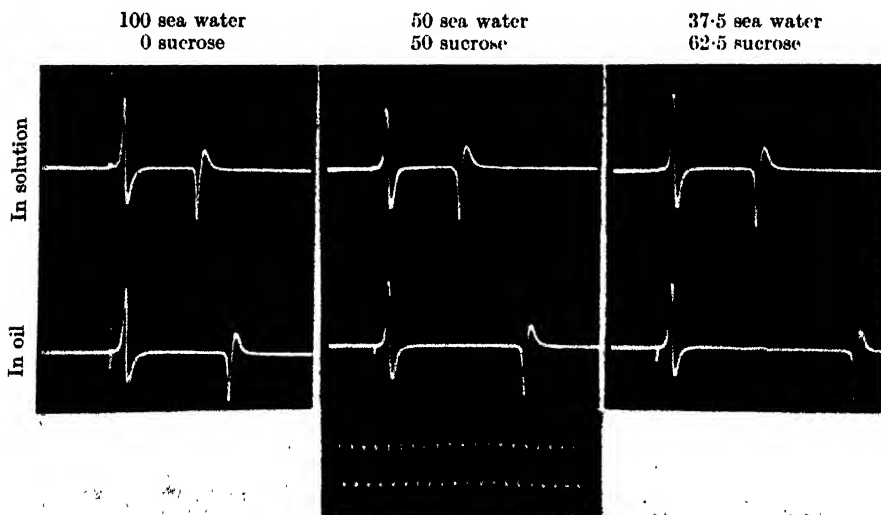
Fig. 2. *Carcinus* nerve fibre at 21° C. 15·5 mm. conduction distance. *Upper* set of records: axon in a large volume of solution. *Lower* set of records: axon in paraffin oil. Time marks: 2000 cyc./sec.

from left to right, it is seen that electrolyte deficiency has only a slight effect on the impulse rate, provided the fibre is kept in a large volume of solution. (ii) There is, however, a very marked reduction of velocity, with increasing sucrose content, if the fibre is surrounded by oil (lower row). Examining each vertical pair of records, we see that a *volume reduction* of the external solution has a much more pronounced effect when the salinity is low. The quantities of the velocity changes are shown in Tables 1 and 2, where they are expressed as

ratios of the conduction times. A summary of several experiments is given in Tables 3 and 5.

TABLE 1. Single *Carcinus* axon. 21° C. Total conduction distance 15·5 mm., corrected for 1·5 mm. conduction 'in oil'. Uncorrected velocity changes are shown in brackets

| | | Conduction times in msec. | | | | Ratio of conduction velocities | |
| | | Uncorrected | | Corrected | | | |
Sea-water/ sucrose volume ratio	Relative resistivity of solution	'Solution'	'Oil'	'Solution'	'Oil'	'Solution' to 'oil'	'Sea water' to 'solution
100/0	1	2·92	4·0	2·52	3·6	1·43 (1·37)	—
50/50	2·46	3·15	5·96	2·62	5·43	2·07 (1·90)	1·04 (1·08)
37·5/62·5	3·39	3·65	8·04	2·98	7·37	2·48 (2·20)	1·18 (1·25)
28/72	4·55	4·5	11·6	3·6	10·7	2·97 (2·58)	1·42 (1·54)
100/0	1	2·94	4·1	2·53	3·69	1·46 (1·40)	—

TABLE 2. Single *Carcinus* axon. 21·5° C. Total conduction distance 14·7 mm., corrected for 1 mm. conduction 'in oil'

| | | Conduction times in msec. (for 13·7 mm.) | | Ratio of conduction velocities | |
Sea-water/ sucrose ratio	Relative resistivity of solution	'Solution'	'Oil'	'Solution' to 'oil'	'Sea water' to 'solution'
100/0	1	2·5	3·14	1·255	—
50/50	2·46	2·64	4·19	1·585	1·055
36/64	3·52	2·76	5·08	1·84	1·105
26/74	5	3·2	6·6	2·06	1·28
18/82	7·3	3·83	9·2	2·40	1·53
13/87	10·75	4·88	Blocked	—	1·95
9/91	14·8	Blocked	Blocked	—	—
100/0	1	2·95	3·66	1·24	—

These results are in agreement with Hodgkin's (1939) findings and, in a qualitative way, might have been predicted on the basis of the electrical theory of propagation (e.g. Rushton, 1937). The results seem accurate enough to justify a somewhat more detailed analysis and a quantitative comparison with theory.

Henceforth the following symbols will be used:

v = impulse velocity.

r_i = resistance of axoplasm.

r_w = effective external resistance, with the fibre in a *large volume of normal sea water*.

r_0 = external resistance, with the fibre surrounded by a *film of normal sea-water* and immersed in *paraffin oil*.

According to the local circuit theory (e.g. Rushton, 1937; Offner, Weinberg & Young, 1940), v is inversely proportional to $\sqrt{(r_i + r_0)}$, hence a change of outside resistance should affect the impulse velocity in the manner described by equation (1)

$$\frac{v}{v'} = \sqrt{\frac{r_i + r_0'}{r_i + r_0}}, \tag{1}$$

27—2

where v and v' are the conduction velocities, r_0 and r_0' respectively being the longitudinal resistances on the outside.

Now, one cannot expect this simple relation to apply to the case of electrolyte withdrawal, for it is unlikely that this would leave the properties of the membrane unaffected. On the other hand, with any given chemical composition of the bath, a change of the conducting outside volume (e.g. transfer from 'solution' to 'oil') might be expected to have no other effect than simply to reduce the external conductance, and to this, equation (1) should apply.

Let us consider the results for *seawater* and $\frac{1}{2}$-*sucrose* shown in Table 1. First, the conduction rate falls by only 4% when the axon is transferred from a large volume of sea water to a large volume of $\frac{1}{2}$-sucrose. In either case the effective external resistance might be regarded as negligible, compared with that of the axoplasm, and the slight drop of velocity might be wholly due to membrane alteration. Alternately, the external resistance may not be negligible, and the 4% change would then be partly, or perhaps entirely, due to an external conductance change. Both assumptions will be examined.

A. If r_w is negligible, we obtain for *sea water*, comparing the velocities in 'oil' and 'solution'

$$\sqrt{[(r_i + r_0)/r_i]} = 1.43, \tag{2}$$

hence $r_0/r_i = 1.05.$

From this, we can calculate the velocity change which should occur on transferring the '$\frac{1}{2}$-*sucrose axon*' from 'solution' to 'oil'. With $\frac{1}{2}$-sucrose (external resistivity being 2·46 times that of sea water) we obtain

$$\sqrt{[(r_i + 2.46r_0)/r_i]} = \sqrt{3.58} = 1.9.$$

Thus the velocity should be reduced to 1/1·9. The observed change was to 1/2·07. In Table 3, the results of seven experiments are shown to which this calculation has been applied. The observed effect is, on the average, slightly greater than the calculated, though the agreement is reasonably good.

TABLE 3. Comparison of observed and calculated velocity changes, from 'solution' to 'oil', with $\frac{1}{2}$-sucrose. r_w is assumed to be negligible

	Ratio of velocities in 'solution' and 'oil'							Mean
Sea water	1·43	1·24	1·28	1·31	1·43	1·255	1·415	1·34
$\frac{1}{2}$-Sucrose: Obs.	2·07	1·50	1·585	1·815*	1·94	1·585	1·90	1·77
Calc.	1·90	1·54	1·61	1·70*	1·90	1·55	1·87	1·725

* In this experiment a 20% hypertonic sucrose stock solution was used.

B. The effective external resistance in a large volume may be appreciable, and although it is difficult to estimate its true value, an upper limit can easily be found. If we assume that the 4% velocity change on transfer from sea water

to $\frac{1}{2}$-sucrose is wholly due to an increase of external resistance, we can find the limiting value of r_w/r_i as follows:

$$\sqrt{\frac{r_i+2\cdot46r_w}{r_i+r_w}}=1\cdot04,$$

hence $\qquad\qquad r_w \leqq 0\cdot058 r_i.$

Using this extreme value of r_w and recalculating the velocity change from 'solution' to 'oil' we find

for sea water $\qquad \sqrt{[(r_i+r_0)/(r_i+r_w)]}=\sqrt{[(r_i+r_0)/1\cdot06r_i]}=1\cdot43,$

hence $\qquad\qquad r_0/r_i=1\cdot17;$

and for $\frac{1}{2}$-sucrose

$$\sqrt{[(r_i+2\cdot46r_0)/(r_i+2\cdot46r_w)]}=\sqrt{(3\cdot88/1\cdot148)}=1\cdot84,$$

which does not differ much from the ratio (1·9) calculated in § A.

With higher concentrations of sucrose, the calculated results are more seriously affected by the assumed value of r_w. As 6 % appears to be an extreme value of r_w/r_i (§ B), the calculations in Tables 4 and 5 were based on two alternative assumptions, (a) that r_w is completely negligible, as argued in § A above, and (b) that r_w/r_i equals 3 %.

TABLE 4. Comparison of observed and calculated effects,
using the results of Tables 1 and 2

Relative saline content (sea water = 100)	Relative resistivity	Ratio of velocities in 'solution' and 'oil'		
			Calculated	
		Observed	(a) $r_w/r_i=0$	(b) $r_w/r_i=0\cdot03$
		Experiment of Table 1		
100	1	1·43	—	—
50	2·46	2·07	1·9	1·87
37·5	3·39	2·48	2·14	2·08
28	4·55	2·97	2·41	2·31
		Experiment of Table 2		
100	1	1·255	—	—
50	2·46	1·585	1·55	1·54
36	3·52	1·84	1·74	1·70
26	5	2·06	1·97	1·90
18	7·3	2·40	2·27	2·14

TABLE 5. Mean values of observed and calculated velocity changes

No. of experiments	Relative saline content (sea water = 100)	Relative resistivity	Mean ratio of velocities in 'solution' and 'oil'		
				Calculated	
			Observed	(a) $r_w/r_i=0$	(b) $r_w/r_i=0\cdot03$
7	100	1	1·34	—	—
7	50	2·46	1·77	1·725	1 695
5	37	3·45	2·06	1·94	1·88
4	28	4·55	2·32	2·18	2·09
3	20	6·45	2·42	2·40	2·27

On the whole, the observed slowing of the impulse 'in oil' is somewhat greater than calculated, though, taking the average values in Table 5, the discrepancies are not striking.

DISCUSSION

As the electrolytes are progressively replaced by sucrose, the impulse is slowed, and eventually blocked, even in a large bath of the solution (Tables 1 and 2). These changes, at least with a salt reduction to less than $\frac{1}{3}$, cannot be accounted for by an increase in external resistivity, even if an improbably high value of r_w/r_i is assumed, and they are presumably due to a change in the membrane properties.

When the axon is transferred from 'water' to 'oil', there is a more marked slowing of propagation, most of which seems to be due to an increase of external resistance. But in some experiments, after fairly drastic salt withdrawal, there appears to be a residual effect which is not fully covered by a simple resistance change. A possible explanation of this discrepancy is provided by recent work of Hodgkin & Huxley (1946). During activity a leakage of potassium from the axoplasm occurs, accompanied by a lowering of the membrane resistance. It is possible that withdrawal of electrolytes disturbs the membrane and causes potassium and perhaps other substances to leak from the fibre until a new steady state is established (see e.g. Höber. 1945; Erlanger & Blair. 1938). If such leakage occurred, it would alter the electrical membrane properties (resting potential, transverse resistance and 'threshold') and, therefore, affect the impulse velocity. Moreover, the leaking substances would accumulate when the fibre is immersed in oil and thus have a greater effect than when it is bathed in a large volume of solution.

Such complicating factors should perhaps be expected when working with salt-deficient, or other abnormal, solutions. It is noteworthy, however, that the anomalous effects do not become appreciable until the salt deficiency exceeds some 60%. There is, therefore, no reason to doubt that in Hodgkin's (1939) experiments, where a normal, balanced, medium was used, the change from 'water' to 'oil' was due entirely to an increase of external resistance.

SUMMARY

1. The effects on conduction rate of electrolyte deficiency and of resistance changes on the outside of a non-medullated nerve fibre (*Carcinus maenas*) are studied.

2. Transferring the axon from sea water to sucrose/sea-water mixtures reduces the conduction velocity progressively as the salinity is reduced.

3. This effect is relatively slight if the fibre is surrounded by a large volume of solution, but very pronounced if the axon is immersed in paraffin oil.

4. Attention is paid to the slowing of conduction on transferring the fibre from a bath of solution to paraffin oil, with any *given* salinity of the medium. Assuming that only the external resistance is affected by this transfer, the slowing of the impulse is calculated from the local circuit theory.

5. There is reasonable agreement between observed and calculated velocity changes, though often the observed changes are somewhat larger.

6. If, in electrolyte-deficient media, a leakage of potassium and perhaps other substances were to occur, which accumulated when the fibre was immersed in paraffin oil, this extra slowing would be explained.

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QUANTITATIVE STUDIES OF ADRENALINE AND NORADRENALINE

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It is fairly generally conceded that the active substance liberated by cholinergic nerves is acetylcholine, but the chemical mediator (or mediators) liberated by adrenergic nerves has not yet been identified with certainty. The evidence of experiments with isolated frogs' hearts and perfused rabbits' ears has supported the view that this substance is adrenaline and not noradrenaline (Loewi, 1936; Gaddum & Kwiatkowski, 1938, 1939; Gaddum, Jang & Kwiatkowski, 1939). On the other hand, Cannon & Rosenblueth (1933, 1937), utilizing methods in which the active material was carried by the blood to produce its effects on distant organs, have given results which are incompatible with the view that adrenaline is the only substance liberated.

The theory that the substance liberated on stimulation of the hepatic nerves in the cat is noradrenaline or some similar substance was put forward by Bacq (1934) and by Stehle & Ellsworth (1937). Greer, Pinkston, Baxter & Brannon (1937, 1938) have also considered noradrenaline as a possible sympathetic mediator, and have compared the effects of hepatic nerve stimulation (liberating presumably pure sympathin E) with those of injections of adrenaline and noradrenaline in the same animal. In a similar series of experiments, Gaddum & Goodwin (1947) found no evidence against the theory that liver sympathin is noradrenaline. In addition, they confirmed the fact that liver sympathin might inhibit the virgin uterus and intestine, a conclusion of considerable importance as it had been assumed that sympathin E was liberated in this organ, and that it was uncontaminated with sympathin I. There is no evidence that sympathin I is ever liberated uncontaminated with sympathin E.

In 1910, Barger & Dale noted that a dose of ergotoxine sufficient to reverse the pressor effect of *dl*-adrenaline in the spinal cat did not reverse that of *dl*-noradrenaline. The pressor response of the latter substance was greater than that of the former, but relaxation of the isolated non-pregnant uterus was slight. Euler (1946 *a, b, c, d, e*) used these properties in demonstrating that extracts of various mammalian organs (except placenta) contained a pressor

substance with properties like those of noradrenaline or dihydroxy-nor-ephedrine (corbasil). He found also that the active material in extracts of frog's heart was identical with adrenaline, so confirming Loewi's conclusion.

In the experiments described below, the activities of the two amines, adrenaline and noradrenaline, were compared quantitatively with one another with the object of devising methods for distinguishing them in extracts and perfusates by parallel quantitative assays. In the adrenal medulla, adrenaline occurs as the laevo-isomer, and by analogy one might expect that, if there are other natural compounds of a similar nature, they would occur as the *l*-isomers. It would have been more satisfactory, therefore, to have compared *l*-adrenaline and *l*-noradrenaline, but the latter compound is not yet available.

METHODS

Chemical. Comparisons were made (a) colorimetrically by the method described by Shaw (1938), and (b) fluorimetrically. For the latter test, 3 ml. of solution and 0·3 ml. of 2 N-NaOH were used to produce maximum fluorescence. This is a modification of Gaddum & Schild's (1934) method, found by West (1947) to give optimal results, when used in a simple fluorescence comparator in conjunction with a Wood's glass to absorb practically all visible rays.

Biological. For experiments on entire animals, spinal cats and cats anaesthetized with Dial Compound (0·85 mL./kg. intravenously) were used. Blood-pressure records were taken from the carotid artery, and injections of the drugs were made into the femoral vein. Contractions of the nictitating membrane were recorded isotonically. For enhancement of the pressor responses, cocaine hydrochloride (8 mg./kg.) was given intravenously; to reverse the adrenaline action, ergotoxine ethanesulphonate (2 mg. kg.) was used. The isolated mammalian tissues used as test objects were the non-pregnant uterus, ileum and duodenum of the cat, rabbit and rat, and the pregnant uterus of the cat and rabbit. The organs were suspended in 30 ml. of Tyrode's solution, oxygenated and maintained at 37° C. For frog tissue experiments, hearts isolated by Straub's method were filled with Clark's Ringer solution. Hearts were also perfused with Ringer's solution by the method of West (1943), improved by the addition of a Mariotte bottle and a special cannula for injection (Gaddum & Kwiatkowski, 1938). The blood vessels of the frog were perfused according to the method previously described (West, 1947).

Solutions of *l*-adrenaline and *dl*-noradrenaline were prepared from the pure bases with N/100-HCl. Samples of *dl*-noradrenaline were kindly supplied by Dr H. Blaschko, Dr U. S. v. Euler and the Sterling-Winthrop Chemical Company.

RESULTS

Chemical tests. Shaw (1938) has shown that catechol derivatives may be active reducing agents in low concentrations, and that small changes in the side chain abolish the effect of alkali. When tested in the present series of experiments, 13·5 μg. of noradrenaline gave a blue colour equivalent to that produced by 1 μg. of adrenaline after alkali treatment. Shaw's figure for the ratio was 16. There was no increase of colour as a result of alkali being added to the noradrenaline before reduction. When treated with alkali for the fluorescence test, noradrenaline produced a green fluorescence at a slower rate and only in stronger solutions than adrenaline (Fig. 1). The ratio of equi-active amounts of noradrenaline and adrenaline was 33·0, a figure roughly agreeing

with that found by Gaddum & Schild in 1934, although these workers made comparisons 20 sec. after the addition of alkali (5N-NaOH).

Intact cat. On entire cat preparations, noradrenaline was always the more active pressor agent, about 10μg. of adrenaline producing the same rise of pressure as 8μg. of noradrenaline (Greer *et al.* 1938). Acceleration of the heart was shown in both cases, but, in many animals, adrenaline caused a 'step' or a depressor notch during the rise of blood pressure, whereas this was almost entirely absent after noradrenaline injections. On the sensitized nictitating
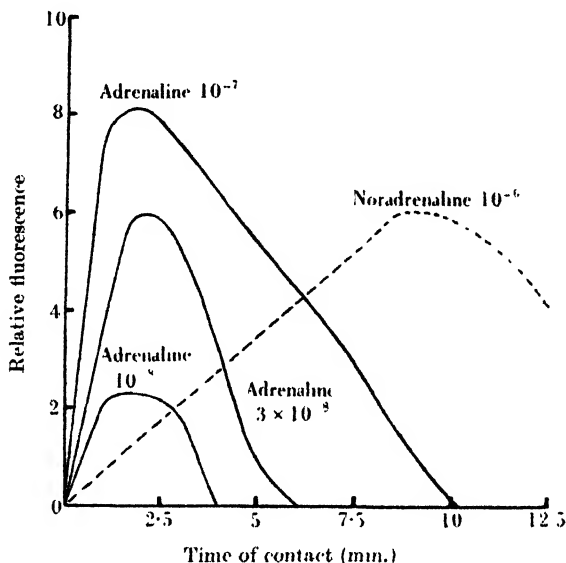
Fig. 1. The fluorescence of solutions of adrenaline and of noradrenaline after treatment with 1/10 vol. 2N-NaOH.

membrane, adrenaline was more active. 8μg. producing a contraction as large as that from a dose of 10μg. of noradrenaline. A dose of ergotoxine, sufficient to reverse the pressor action of adrenaline, decreased but did not reverse the action of noradrenaline. Both actions were enhanced by cocaine.

Isolated mammalian tissues. Most of the comparisons are recorded in Table 2, the figures representing the average of at least six determinations on each fresh tissue (stored 0 days). Both drugs caused relaxation of these tissues, except the rabbit non-pregnant uterus where both caused contraction. Values found in experiments with the duodenum agreed well with those listed for the ileum. When compared with adrenaline, noradrenaline was found to be more powerful an agent on the gut than on the non-pregnant uterus. The ratio on the rat uterus is interesting and is different from previous observations (Greer *et al.* 1938). In nearly all preparations, including those where the responses were motor, the action of noradrenaline had a longer latent period than that of

adrenaline (Fig. 2) and the return to normal tone was much slower, two washings of the tissue being required for complete recovery. Ratios for isolated pregnant uteri of the cat and rabbit are recorded in Table 1. On the former,

TABLE 1. Ratio of dose of *dl*-noradrenaline to equi-active
dose of *l*-adrenaline on various fresh preparations

Test object	Ratio	Excitor (*E*) or inhibitor (*I*) action
Cat, blood pressure	0·8	*E*
Cat, pregnant uterus	0·8	*E*
Cat, ileum	1·0	*I*
Cat, nictitating membrane	1·25	*E*
Rabbit, ileum	2·0	*I*
Rat, ileum	3·0	*I*
Rabbit, pregnant uterus	4·0	*E*
Rabbit, non-pregnant uterus	5·0	*E*
Frog, blood vessels	5·0	*E*
Frog, Straub heart	8·0	*E*
Cat, non-pregnant uterus	10·0	*I*
Frog, perfused heart	33·0	*E*
Rat, non-pregnant uterus	100·0	*I*
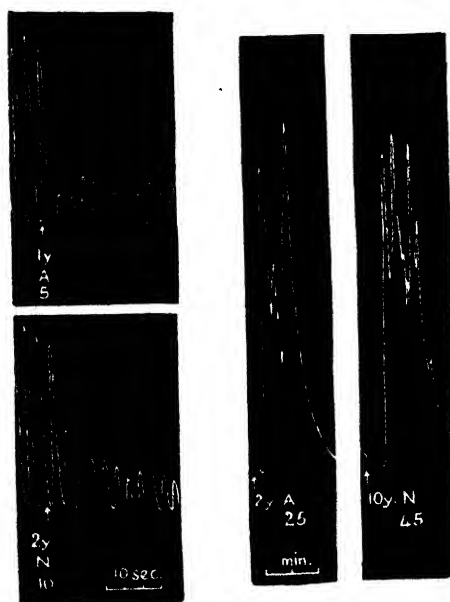
Fig. 2. The effect of equi-active doses of *l*-adrenaline (*A*) and *dl*-noradrenaline (*N*) on rabbit ileum (left tracings) and on rabbit non-pregnant uterus (right). Numbers below doses represent latent periods in sec. Note that noradrenaline possesses a longer latent period than adrenaline in both preparations (i.e. inhibitor and excitor actions).

adrenaline was excitor, but noradrenaline was even stronger (Euler, 1946*c*), 4 μg. of noradrenaline producing a contraction equivalent to that from 5 μg. of adrenaline. On the latter, both produced pure contractions and the ratio was little changed from that found with the non-pregnant preparation. So far,

moderate doses of noradrenaline have never produced a fall in tone in the pregnant preparation, as reported by Euler (1946e). This fact is important, since Labate (1941) showed that the pregnant rabbit's uterus is excited by nerve stimulation and by adrenaline, and that there is no accentuation of either effect after cocaine. Experiments with all these tissues suspended in Locke's solution have produced similar results.

Storage of isolated mammalian tissues. Several workers have shown that storage of isolated organs affects the sensitivity and movements of these structures when set up in the ordinary organ bath. As long ago as 1910, Barger & Dale reported that the virgin cat's uterus in the fresh state was inhibited by p-hydroxyphenylethylamine, but after 3 days' storage in the ice-box it had lost its sensitiveness to most bases with one phenolic hydroxyl group. Ambache (1946) showed that 4 days' cooling of the rabbit jejunum abolished the pendulum movements and the response to potassium, barium and histamine, but did not abolish the response to electrical stimulation and acetylcholine. The response to adrenaline was not included in his series of experiments. By a chance observation, a piece of stored cat ileum was used for obtaining a noradrenaline: adrenaline ratio, and noradrenaline was found to be the more active. Further work, therefore, was carried out to investigate this phenomenon, the results of which are shown in Table 2. All the organs were stored for the prescribed period in Tyrode's solution in the refrigerator (0°–4° C.), and then cut into convenient lengths and warmed to body temperature in the organ bath. The doses of each drug producing a just submaximal inhibition or contraction are recorded in Table 2. The results suggest that the organs, especially the ileum, become less sensitive to adrenaline on storage, the sensitivity to noradrenaline remaining about constant.

TABLE 2. The effect of storage of mammalian organs on their sensitivity to *dl*-noradrenaline and *l*-adrenaline

		Dose of drug producing a just submaximal inhibition or contraction						Ratio of equi-active doses		
		dl-noradrenaline (μg.)			*l*-adrenaline (μg.)					
Animal	Storage of tissue (days)	0	3	5	0	3	5	0	3	5
Cat	Ileum	2	1·5	2·5	2	4	10	1	0·4	0·3
	Non-pregnant uterus	10	10	10	1	1	5	10	10	2
Rabbit	Ileum	2	2·5	3	1	5	15	2	0·5	0·2
	Non-pregnant uterus	10	10	10	2	2	2	5	5	5
Rat	Ileum	3	3	4	1	3	8	3	1	0·5
	Non-pregnant uterus	10	8	15	0·1	0·2	0·5	100	40	30

Frog tissues. On the heart, prepared by Straub's method, noradrenaline was less active than adrenaline, ratio values of 8·0 consistently being recorded.

With perfused hearts, noradrenaline was even less active, the average ratio being 33 : 1. That this alteration was partly due to the speed of penetration and action was shown by perfusing hearts at greatly reduced pressures. With slower outflows, adrenaline was found to be only twenty to twenty-five times as active as noradrenaline. It should be pointed out that, in the Straub preparation, the drug is in contact with the heart muscle continuously, whereas in the perfused heart the drug is injected into the cannula and washed through the heart. It is of interest to note that all workers have agreed that the active substance in a frog's heart is identical with adrenaline (Euler, 1934; Loewi, 1936; Shaw, 1938; Euler, 1946e). When tested on perfused frog blood vessels, five times as much noradrenaline was needed to give vasoconstriction comparable with that produced by adrenaline. Summer frogs needed larger doses than winter ones, but the ratio did not alter.
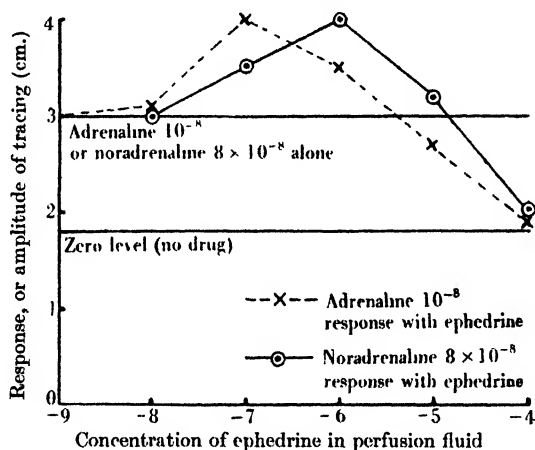
Fig. 3. The influence of ephedrine on the *l*-adrenaline and *dl*-noradrenaline responses in the Straub frog heart. Note that ephedrine is synergistic in low and antagonistic in higher concentrations with both drugs.

Action of ephedrine on responses of frog tissues. Gaddum & Kwiatkowski (1938) showed that ephedrine in low concentrations sensitizes the rabbit's ear, the cat's nictitating membrane and the frog's heart not only to adrenaline, but also to the stimulation of adrenergic nerves. Jang (1940) and Graham & Gurd (1941) used the spinal cat in addition to the above test organs, and obtained clear evidence that ephedrine was synergistic in low and antagonistic in higher concentrations with adrenaline on these preparations. This work has been confirmed on the Straub heart (Fig. 3), the perfused heart and the perfused frog's blood vessels, and extended to include the effect of varying concentrations of ephedrine on the noradrenaline response. It will be seen that sensitization and antagonism of the noradrenaline response run quite parallel with the effects on the adrenaline action, and suggest that adrenaline and noradrenaline utilize

the same receptor substances. It is well known that both drugs are readily and rapidly destroyed by amine oxidase.

Full results for the comparisons on all tissues studied are presented in Table 1. and serve as a guide for the detection of either substance in extracts and perfusates. It is interesting to note that high ratios can be obtained for both excitor and inhibitor actions.

DISCUSSION

In agreement with previous workers, noradrenaline has been found to be more effective than adrenaline as a pressor agent on mammalian tissues, but contrary to Barger & Dale, its inhibitory properties are not always slight (see ileum results). Several values for the ratio of activity have been quoted in the past (Tainter, 1931; Crimson & Tainter, 1938, 1939; Morton & Tainter, 1940), but they have not been consistent. In tissue perfusion experiments, moreover, these workers obtained, with Locke's solution as the perfusion fluid, a ratio quite different from that obtained when blood was perfused. However, with the doses used in the experiments described here, noradrenaline almost always had a longer latent period and a longer recovery time than adrenaline, even if the action was excitor. When added to adrenaline, noradrenaline either had no effect or a simple additive action on the adrenaline response. It is suggested, therefore, that it competes with adrenaline for the receptor substance (as shown by the ephedrine sensitization), but the combination is slow and its high activity prevents its use in concentrations of 100–1000 times that of adrenaline (Jang, 1940; Lawrence, Morton & Tainter, 1942); such concentrations of certain other sympathomimetic amines (e.g. ephedrine) would antagonize the adrenaline effects. It has been suggested that the effect of storage on the sensitivity of tissues to drugs is due to death of the nerves. Such a change would be expected to lead to enhanced responses to adrenaline, sympathin and certain other sympathomimetic amines, but the only effect observed was a diminished response to adrenaline.

The three actions on frogs' tissues shown in these experiments are all excitor, and indicate that noradrenaline is less active than adrenaline. It is possible that frogs' tissues utilize adrenaline for the actions of adrenergic fibres.

The most useful tests for deciding whether the activity of an extract or perfusate is due to noradrenaline or adrenaline are those where the ratio of equiactive amounts is high. The following have been found to be the most suitable: (a) the fluorescence test, detecting 10^{-8} adrenaline and having a ratio of 33·0; (b) Shaw's chemical test, also detecting 10^{-8} adrenaline with a ratio of 13·5; (c) a Straub heart preparation detecting 10^{-9} adrenaline and having a ratio of 8·0; and (d) the perfused frog blood-vessel preparation which detects 10^{-7} adrenaline and has a ratio of 5·0. The value of quantitative agreement between these different tests in the identification of pharmacologically active substances has been shown by Chang & Gaddum (1933).

SUMMARY

1. By means of chemical and biological tests, the activity of *l*-adrenaline has been compared with that of *dl*-noradrenaline. It has been confirmed that the latter substance is a stronger pressor agent and has a more powerful motor effect on the pregnant cat's uterus than adrenaline, but in all the other tests studied it is weaker in action.

2. Storage of mammalian intestine results in a decreased sensitivity to adrenaline but not to noradrenaline.

3. The action of noradrenaline on the frog's heart, like that of adrenaline, is increased by small doses of ephedrine.

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THE ESTIMATION OF ADRENALINE
IN NORMAL RABBIT'S BLOOD

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Many attempts have been made in the past to determine the concentration of adrenaline in blood and other fluids, but suitable quantitative methods are few. Using biological test organs, Stewart & Rogoff in 1922 found that the normal adrenaline content of blood from the carotid artery was 1 in 10^9, whilst that in the suprarenal vein was 2 in 10^6. Whitehorn (1935), using a chemical method and silicic acid for adsorption, claimed to estimate adrenaline in blood in a concentration of $0·02\mu g./ml.$ Later, Shaw (1938) published a modification of this method which is simpler and more sensitive. He found that rabbit's blood had an apparent adrenaline content of $0·05-0·06\mu g./g.$, and most of it probably was adrenaline, as shown by the specific colour increase with alkali. His results with human blood were mostly due to another catechol derivative, as there was little or no colour increase with alkali. Later work (Bloor & Bullen, 1941; Raab, 1943) suggested that adrenaline circulates in the blood of man in very small amounts only.

In recent years, attention has been centred on the fluorescence method of estimating adrenaline in solution (Gaddum & Schild, 1934), and it has been applied to blood (for references see Jørgensen, 1945). The work described below is a modification of Jørgensen's technique, involving the dialysis of haemolysed blood and the subsequent analysis of the dialysate. By using parallel quantitative assays and comparison figures for the activities of adrenaline and noradrenaline (West, 1947b), it has been possible to establish which of these two substances is present in normal rabbit's blood.

METHOD

The method of Jørgensen was followed throughout with very slight modifications. The dialysis was carried out in small circular perspex dishes, held together by a metal clasp and each possessing an outlet, closed by a small screw, for introduction or withdrawal of the fluids. The cellophane membrane separating the two halves had a thickness of 25μ. Experience showed that no vaseline was required on the ground edges. Into one side of the dialyser was introduced 5 ml. of blood and 5 ml. of diluent (N/100-HCl containing 0·1 % glycine, which caused haemolysis); into the other,

5 ml. of diluent and 2 ml. of Shaw's aluminium hydroxide suspension (to remove traces of other fluorescent substances which pass through the cellophane membrane). The dishes were placed on edge and shaken at the rate of about 120 horizontal movements per min. 5 ml. of the recipient fluid were removed by pipette and centrifuged. 3 ml. of the supernatant solution were transferred to a Monax test-tube, 0·3 ml. of 2N-NaOH added, and the fluorescence compared with a standard eosin solution by two methods as previously described (West, 1947a). Shaw's method was applicable also, but the presence of some soluble aluminium salts inactivated the biological test preparations. This was overcome for the Straub frog heart and frog blood-vessels particularly, by omitting the aluminium suspension from the dialyser and diluting the dialysate before testing.

RESULTS

To determine the efficiency of the method, pure adrenaline solutions were employed in place of the blood. Dialysis was carried out for given times, after
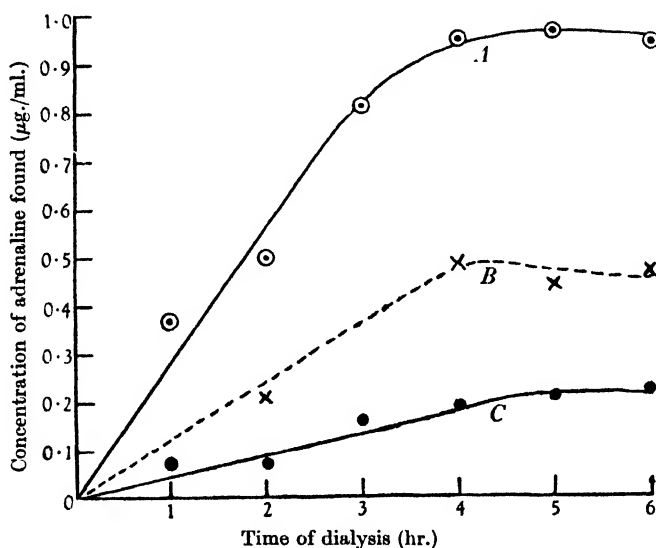
Fig. 1. Recoveries of adrenaline in solution with varying times of dialysis. Concentration of solution $A = 1·0\,\mu g./ml.$, $B = 0·5\,\mu g./ml.$, and $C = 0·2\,\mu g./ml.$ Optimum values were reached after 4 hr.

TABLE 1. The recovery of adrenaline in aqueous solution, using 5 ml. quantities and dialysis for 4 hr.

Weight of adrenaline found ($\mu g.$)

| Concentration of adrenaline ($\mu g./ml.$) | Weight of adrenaline taken ($\mu g.$) | Fluorimetric | | Physiological | | Chemical |
		Jørgensen	West	Straub heart	Frog blood vessel	Shaw
1	5	4·7	4·9	4·7	5·4	4·8
0·2	1	0·86	0·94	0·88	—	1·06
0·1	0·5	0·45	0·47	0·46	0·5	0·61
0·03	0·15	0·18	0·20	0·14	0·2	—

which the dialysate was tested fluorimetrically, chemically and physiologically against the adrenaline standard solution. The results, recorded in Fig. 1,

confirmed that dialysis for 4 hr. was optimal. Recoveries were good with amounts greater than 0·03μg./ml. (Table 1). The biological tests gave results which agreed closely with those obtained fluorimetrically and chemically, each value in the table being the mean of six readings. A few estimations with solutions of noradrenaline showed correspondingly good agreement, though of course much larger amounts had to be used, since noradrenaline is less active than adrenaline on all the preparations tested.

Blood was then taken from the rabbit's ear vein direct into solid sodium citrate (Jørgensen, 1945) and assayed. Quantitative agreement between the different tests was shown when results were calculated as adrenaline (Table 2), although chemical values were always slightly higher than those found by fluorimetric and physiological methods. Chang & Gaddum (1933) indicated the value of such agreement in the identification of pharmacologically active substances. The average of forty-nine determinations of the adrenaline content of rabbit's blood, made on two samples taken from each of six rabbits was found to be 0·098μg./ml., a value which agrees with that of 0·074μg./ml. found by Jørgensen. The few normal human and cat's blood samples that were assayed indicated a smaller amount of adrenaline, values of between 0·03 and 0·05μg./ml. being detected. The differences in the results with different species may have been due to the emotional response of the rabbits to the bleeding.

TABLE 2. The concentration of adrenaline (μg./ml.) in normal rabbit's blood
Dialysis for 4 hr.

| | Method of assay | | | | | | |
| | Fluorimetric | | Physiological | | Chemical | | |
Rabbit	Jørgensen	West	Straub heart	Frog blood vessel	Shaw	Average value	Shaw's specific test
A	0·110	0·094	0·098	0·090	0·120	0·102	2·1
B	0·119	0·122	0·100	0·120	0·127	0·118	2·3

The addition of known amounts of adrenaline to assayed blood of two rabbits produced slightly variable results (Table 3). Added noradrenaline likewise gave slightly lower values than expected, but generally recoveries were reasonable. Shaw's specific test, indicative of adrenaline or sympatol (but not noradrenaline), was positive when applied to blood dialysates, ratio values of more than 2·0 being recorded (Table 2). Experiments on rabbit's red blood cells and plasma suggested that the majority of the adrenaline is carried in the cells (Bain, Gaunt & Suffolk, 1937), since the amount in rabbit's plasma was unmeasurable.

The results shown in Fig. 2 are from two rabbits which were fed on a fairly constant diet. Samples of blood were taken from these animals about the same time (10 a.m.) each day for 5 days, and then 2 mg. adrenaline was injected

subcutaneously in two doses ½ hr. apart. Immediately after the second injection, another sample of blood was taken and yet another at the end of 1 hr. All the samples were assayed for their adrenaline contents by the fluorimetric and biological methods. A rise in blood adrenaline was shown, but the increase had disappeared after 1 hr., thus indicating a fast inactivation process.

TABLE 3. The recovery of adrenaline added to 5 ml. quantities of normal citrated rabbit's blood. Dialysis for 4 hr.

Blood	No. of samples	No. of assays	Mean adrenaline content (µg./ml.)	Adrenaline added to vessel (µg.)	Theoretical concentration (µg./ml.)	Concentration found (µg./ml.)	No. of samples	No. of assays
A	3	12	0·122	1·0	0·322	0·361	3	9
				0·5	0·222	0·257	3	10
B	3	10	0·072	1·0	0·272	0·269	3	10
				0·5	0·172	0·168	3	10
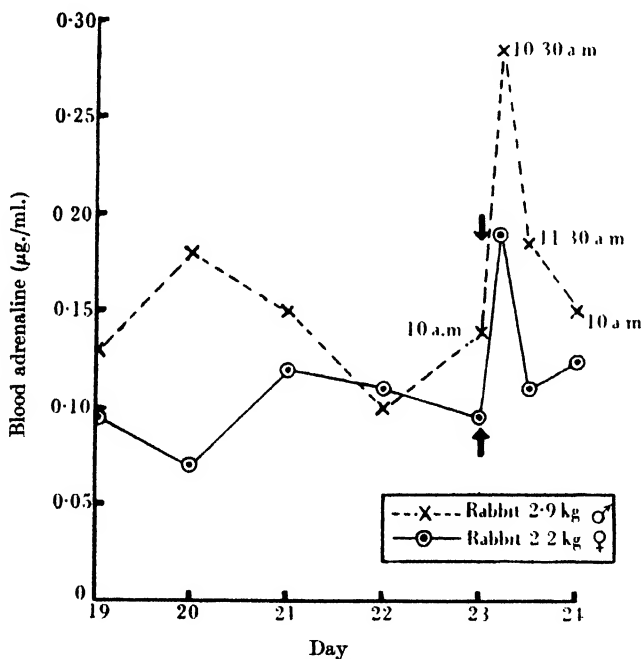
Fig. 2. The effect of 2 mg. adrenaline injected subcutaneously (at the arrows) on the blood adrenaline of two rabbits. After 1 hr. the rise had disappeared. Each point represents the mean of values obtained by the fluorimetric, Straub heart and perfused frog blood-vessel methods.

DISCUSSION

By means of parallel quantitative assays and comparison figures for the activities of adrenaline and noradrenaline, normal rabbit's blood has been found to contain adrenaline at a concentration of 0·098µg./ml. On no occasion have the assays indicated the presence of noradrenaline, since quantitative

agreement between the different tests was shown when the results were calculated as adrenaline. The proof that rabbit's blood contains adrenaline and not noradrenaline is important, since the work of Euler (1946*a, b, c*) showed that extracts of mammalian spleen, heart and sympathetic nerves contain an active substance similar in action to noradrenaline. It adds weight to the suggestion that noradrenaline might be the precursor of adrenaline (Blaschko, 1942), and that methylation might occur on stimulation, the methyl group being derived from the choline constituent of lecithin. Examples of biological transmethylation are quite numerous, methionine and choline usually acting as donors of the methyl group.

SUMMARY

1. By means of chemical, fluorimetric and physiological methods, it has been possible to demonstrate the presence of adrenaline (and not noradrenaline) in normal rabbit's blood.

2. The average concentration in duplicate blood samples from six rabbits was 0.098μg./ml., and this value was nearly doubled after 2 mg. of adrenaline were injected subcutaneously.

3. The possible relation of noradrenaline and adrenaline to the mediator liberated by adrenergic nerves is discussed, and transmethylation of noradrenaline suggested.

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INULIN, DIODONE, CREATININE AND UREA CLEARANCES IN NEWBORN INFANTS

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Although it is generally agreed that the human kidney at birth is immature, the nature of the immaturity, in spite of its obvious practical importance, has not yet been fully determined. On the anatomical side it has been known for many years that, in foetal life, the glomerular tufts are covered by a layer of thick epithelial cells (Klein, Burdon-Sanderson, Foster & Brunton, 1873), and it has been suggested by Gruenwald & Popper (1940) that this covering ruptures suddenly at birth. Potter & Thierstein (1943) have shown that glomerular units are still being developed as late as the 36th week of intra-uterine life. Work has also been carried out on the functional immaturities which are a part of the early days of life. Thus, the inulin clearances, the urea clearances, and also the sodium, chloride and potassium clearances have all been found to be low when compared with those of adults on a basis of surface area (Barnett, 1940; Barnett, Perley & McGinnis, 1942; McCance & Young, 1941). Our knowledge of this subject was briefly reviewed by McCance (1946), but there was little or nothing to be found at that time about the function of the tubules. It seemed advantageous, therefore, to make studies of tubular activity, and this paper records simultaneous measurements of inulin, urea, diodone and creatinine clearances in newborn children.

METHODS

Since it was necessary to collect several comparatively large samples of blood, and to catheterize the babies for several hours, it did not seem justifiable to work with normal children, and the experiments described here were made on full-term infants who were born with inoperable meningo-myelocoeles. These babies all had complete functional disorganization of the spinal cord in the lumbar region. Although such children have a very short expectation of life, it was considered that they might reasonably be supposed to have normal renal function for a few days after birth, and, when in due course they died, post mortem examinations in fact failed to reveal any renal abnormality. The congenital defects that usually accompany meningo-myelocoeles were, however, found. Thus, there was always an internal hydrocephalus, and irregularity in the formation of the

bones in the skull giving rise to lacunae or fenestration. One child had accessory digits on hands and feet, and other children had bilateral talipes. All the children were apparently in good general condition at the beginning of the experiments and appeared to be unharmed by them.

Seven babies have been studied, and some of the relevant information about their age, sex, weight and surface area will be found in Table 1.

TABLE 1. Sex, age, weight and surface area of the newborn children
used for clearance experiments

Initials	Sex	Age (days)	Weight (g.)	Surface area (sq.m.)	Correction factor (1·73/surface area in sq.m.)
W.E.	M.	2	2240	0·181	9·5
F.L.	M.	7 and 8	2980	0·216	8·0
G.T.	F.	2	4040	0·268	6·4
M.P.	M.	2	2200	0·179	9·7
A.B.	M.	4	2970	0·215	8·0
B.P.	F.	3	3150	0·225	7·7
B.B.	F.	2	3960	0·264	6·5

Each child was placed on its side in its cot for the experiment on a high pillow arranged so that catheter drainage was made easier by the collecting vessel being on a lower level than the child. A cannula was then inserted into one of the internal saphenous veins at the ankle, and 100–150 c.c. of a solution containing 0·18 % NaCl and 4 % glucose was given over a period of about 1 hr. by the usual 'drip' and gravity method. It was not necessary to splint the leg, or to use a local anaesthetic for the insertion of the cannula because these children had no power of voluntary movement, and had no appreciation of pain. Before the end of the hour the flow of urine usually increased, and the infusion of glucose-saline was stopped as soon as it was obvious that a diuresis had been established. The amount of glucose given in this way was never more than 6 g. and, when tested, the urine was always found to be free from sugar. If the amount of fluid had been correctly judged, the diuresis fell off gradually during the experiment. If too much was given, however, the urine volumes did not fall but continued to rise. This happened in studying the child B.P., but in extenuation it may be said that no two children were alike in the speed at which they excreted the fluid given, and it was not possible to make an accurate prediction of the response to the infusions.

While the infusion was being given, a catheter, lubricated with sterile liquid paraffin, was inserted into the bladder. A soft rubber catheter, size 3, was used for the girls, and a bicoudé gum-elastic catheter, size 4, for the boys, and the use of these comparatively large sizes helped to prevent leakage, which was never troublesome. The catheters were passed without much difficulty and did not cause any visible trauma, or any excessive reaction from the child. One boy had a very tight prepuce which was stretched so that the meatal opening could be seen, but it was later discovered that it was possible to catheterize such children 'blind'. The catheter was fixed to the thigh of the leg into which the infusion was running, and its end was inserted into a small graduated measuring cylinder. Usually the catheter emptied the bladder completely, but at the end of each experimental period pressure was applied to the abdominal wall. 'Bimanual' pressure, with a finger of one hand in the child's rectum and the other hand on the abdominal wall, was found to be no improvement on simple supra-pubic pressure. It was often necessary to adjust the catheter many times before a continuous stream of urine could be obtained, small amounts of from 2 to 3 c.c. appearing intermittently in the cylinder if the adjustment was not exact.

When the urine flow was satisfactory, a 'priming' solution, containing inulin, diodone and creatinine in amounts sufficient to provide adequate blood concentrations, was given in 1 to 2 min. by syringe into the cannula in the vein. It was immediately followed by the 'maintenance' solution which was given by gravity, and which was intended to keep the concentrations of these substances at a steady level in the blood. The quantities which were found by trial to be satisfactory are given in Table 2.

TABLE 2. Composition of priming and maintenance solutions

	0·9% NaCl (c.c.)	Inulin (g.)	Diodone (c.c.)	Creatinine (g.)
Priming	20	0·40	0·20	0·12
Maintenance	200	5·00	2·00	2·00

Note. These are the amounts which were found to be satisfactory. They were arrived at by trial and error and they were not used in all experiments.

For an experiment on a child weighing 2750–3500 g., 10 c.c. of the priming solution were given, and the maintenance solution was run in at the rate of about 20 c.c./hr. The amounts were varied in accordance with the body weights. After the maintenance infusion had been running for about 20 min. the bladder was emptied and the first experimental period began. After three or four such periods the plasma diodone was raised by the rapid injection of 3–5 c.c. of undiluted diodone solution into the vein through the cannula. After this, the maintenance solution was given more rapidly for a few minutes, the interim urine was discarded and then urines and bloods were collected for two or three more experimental periods.

The inulin was purified in the first place by boiling with charcoal and precipitation with alcohol, as recommended by Smith, Chasis & Ranges (1937–8). Before its administration to the babies, the partially purified inulin was dissolved in very hot water, and the hot solution was then passed through a Seitz filter, fitted with a fine pad, designed to exclude bacteria. This second purification seems to be necessary to remove the last of the pyrogens. The creatinine was added to the filtered solution, which was then boiled for a few minutes, and finally, when the temperature had fallen to about 40° C., the diodone was added. Bayers Ltd. have now stated that 'Perabrodil', the proprietary brand of diodone used for these experiments, can be boiled without fear of decomposition, so that the precaution of adding the diodone after the solution had cooled was probably unnecessary.

The blood samples were obtained from the anterior fontanelle by needle and syringe, and each was transferred immediately to a centrifuge tube containing heparin, and gently stirred with a thin glass rod. The blood was centrifuged for 20 min. at about 1200 r.p.m., and the plasma separated without delay. The samples were taken half way through the period over which urine was being collected. These periods varied in length so that in each a reasonable amount of urine (at least 3–4 c.c.) was obtained. To avoid the possibility of the inulin not remaining in solution in the urines, 10 c.c. of distilled water were added to each specimen as soon as it had been measured. Toluene was used as the preservative.

Cadmium sulphate was used to precipitate the plasma proteins, and fresh filtrates were made for each estimation. Bloods and urines were always estimated in duplicate in the same batch, so that the clearances might be more exactly determined, even if errors of technique should produce different absolute levels in different batches.

The method of White & Rolf (1940), modified on the lines suggested by Bak, Brun & Raaschou (1943) was used for the estimation of diodone. Inulin was estimated by a modification of Roe's (1934) method, proposed by S. W. Cole (to be published); and creatinine by the Jaffé reaction, in the manner described by Folin (1914). Urea was estimated by the methods of Lee & Widdowson (1937) and of Archibald (1945), these two methods giving very satisfactory agreement when used for the analysis of identical samples.

Two incidental findings may perhaps be mentioned here. The inulin method mentioned above depends on the hydrolysis of the inulin to fructose, and the determination of the fructose by the colour it produces with resorcinol. Attention has recently been directed to the presence of fructose in the foetal tissues of several species, and Cole & Hitchcock (1946) have shown that in the sheep this fructose disappears very soon after birth. In several of these experiments blood was taken before any inulin had been given, but no measurable amounts of fructose were found in these bloods, and it seems, therefore, that, if this sugar is present in the human foetus, it disappears rapidly after birth.

The second finding of interest was that small quantities of inulin could be demonstrated in fluid aspirated from the meningo-myelocoele sacs, having presumably passed the meningeal barrier.

Adult controls. The clearances of inulin, creatinine, urea and diodone in adult men and women have been measured by a number of people, and their general levels and relationships have now been established. Inulin clearances in normal persons average about 120–130 c.c./min. and creatinine clearances are higher so that the creatinine/inulin clearance ratios are of the order of 1·3–1·5. Raising the level of creatinine in the plasma depresses the creatinine clearance, and it is considered that in man some creatinine is excreted by the tubules, although most of it finds its way into the urine by glomerular filtration. Diodone clearances vary from 450 to 900 c.c./min., provided the level of diodone iodine in the plasma does not exceed about 5 mg./100 c.c. When the amount of diodone iodine in the plasma is raised above this figure the clearances decline because the tubules can no longer remove all the diodone from the blood passing by them. They do, however, excrete more and more diodone till their capacity to excrete the compound is completely saturated. The amount of diodone excreted by the tubules per min. under these circumstances may be measured and has been used as an index of tubular activity, and of tubular mass ($Tm.$). The urea clearances are always lower than the inulin clearances and vary with the minute volume of the urine. It is considered that some of the urea filtered off in the glomeruli escapes back from the tubules into the blood.

Table 3 gives some results which have been obtained on adults with normal kidney function. These tests have been made at different times and for various reasons. The materials and methods used for the adult work have, however, been exactly the same as those employed for the baby experiments except that the diuresis was obtained by giving water by mouth. It will be seen that the results are in line with those of previous reports, and it may be assumed that satisfactory techniques have been employed.

The newborn infants. Table 4 gives the results which have been obtained. For purposes of comparison, the minute volumes and clearances of the infants have been multiplied by a factor relating their surface area to that of the standard adult, who is assumed to have a surface area of 1·73 sq.m. The factor has been calculated in accordance with the recommendations of Howland & Dana (1913). A discussion of the relative merits of this and other means of comparing infants with adults will be found in the paper of McCance & Young (1941). Although it is obviously necessary to have some basis of comparison, the use of any factor is somewhat arbitrary, and it is always satisfactory to be able to make internal comparisons, by studying clearance ratios, for example, which involve no factors.

It has been shown (Barnett, Perley & McGinnis, 1942; McCance & Young, 1941) that in infants the inulin and urea clearances may vary with the volumes

TABLE 3. Clearances of normal adults for comparison with those of newborn children

Initials	Min. vol. (c.c.)	Inulin clearance (c.c./min.)	Diodone clearance (c.c./min.)	Diodone inulin clearance ratio	Creatinine clearance (c.c./min.)	Creatinine inulin clearance ratio	Urea clearance (c.c./min.)	Urea inulin clearance ratio
W.L.	10·4	122	465	3·8	164	1·34	76	0·62
E.W.	5·7	120	482	4·0	159	1·32	74	0·61
H.B.	13·5	123	420	3·4	160	1·30	94	0·76
M.W.	7·4	132	505	3·8	164	1·24	90	0·68
R.W.	11·9	129	575	4·5	170	1·30	84	0·65

Note. The clearances shown are the average of results obtained from two experimental periods.

TABLE 4. Clearances of newborn children

	Inulin			Diodone			Creatinine			Urea		
Initials	Min. vol. (c.c./1·73 sq.m./min.)	Plasma (mg./100 c.c.)	Clearance (c.c./1·73 sq.m./min.)	Plasma (mg. diodone iodine/100 c.c.)	Clearance (c.c./1·73 sq.m./min.)	Diodone inulin clearance ratio	Plasma (mg./100 c.c.)	Clearance (c.c./1·73 sq.m./min.)	Creatinine inulin clearance ratio	Plasma (mg./100 c.c.)	Clearance (c.c./1·73 sq.m./min.)	Urea inulin clearance ratio
W.E.	0·68	47·9	16·3	4·40	44·0	2·7	61·0	12·9	0·86	42·0	5·5	0·41
F.L.	3·40	20·8	43·6	2·59	87·8	2·13	4·3	57·0	1·12	31·5	27·2	0·63
F.L.	1·68	44·8	23·3	4·18	90·4	3·37	12·4	34·6	1·32	25·1	16·2	0·77
G.T.	1·46	91·7	18·2	7·12	25·2	1·39	20·5	18·2	1·13	22·9	12·1	0·41
M.P.	2·82	114·5	16·9	16·62	23·5	1·40	22·6	12·8	0·79	21·8	8·8	0·54
A.B.	2·54	52·6	44·6	0·72	124·7	2·83	13·1	37·8	0·87	22·0	18·2	0·38
B.P.	3·15	67·0	29·5	5·17	71·5	2·42	18·3	22·3	0·76	52·5	14·2	0·48
B.B.	1·09	41·6	25·1	1·37	54·4	2·46	17·8	15·1	0·68	41·0	14·4	0·65
Average ratios of all children						2·34			0·98			0·53

Note. This table shows averaged results of three or four experimental periods in each case. The ratios shown are averaged ratios, calculated by adding the ratios found in the individual periods, and dividing by the number of periods. The average ratios shown in the bottom line were obtained in the same way.

of urine passed per minute, and the main purpose of including figures for the averaged minute volumes is to demonstrate that they were reasonably high—in other words, that oliguria was not responsible for any low clearances which may have been observed. The inulin clearances were all very low by adult standards. This is not a new observation, but the results confirm those of Barnett (1940), Barnett et al. (1942), and McCance & Young (1941). Baby F.L. was tested on two consecutive days, and the inulin clearances were nearly twice as high on the first day as they were on the second. It may be that glomerular filtration rates vary from day to day much more in infants than in adults, and vary for reasons that are at present not appreciated. The diodone clearances were also very small. The child M.P. was given too much diodone but the results obtained from the other children show that at plasma diodone iodine levels of 0·72–7·12 mg./100 c.c. the clearances were never over 125 c.c./min./1·73 sq.m., and in most instances were very much lower. This is far below the range which is accepted as being normal for adults, and furthermore the diodone/inulin clearance ratios were never up to adult levels even when the plasma iodine concentrations were below 2 mg./100 c.c.

It is difficult to know what to make of these results in terms of adult renal physiology. The low glomerular filtration rates will in part explain the low diodone clearances. It is generally considered, however, that in adults the tubules excrete diodone so efficiently that the diodone clearances at low levels of plasma diodone can be used to measure the effective blood flow through the kidney. Apart from the possibility of arterio-venous anastomoses it would clearly be unjustifiable at present to make the same assumption about infants, and the evidence is really against it. Thus, the anatomical evidence suggests that the glomeruli are likely to be less rather than more permeable at birth than they are in adult life, and this would indicate that a smaller proportion of the plasma, passing through the glomeruli, was likely to be filtered off in infants than in adults. Yet, if the diodone clearances are interpreted as measuring the renal plasma flow, the inulin/diodone clearance ratios observed suggest that the filtration fractions in babies are very high. This is unlikely, and it is much wiser at present to interpret the results as indicating that the blood is not completely cleared of diodone in one passage through the kidney. It has been suggested that the diodone clearances may have been reduced because the tubules were actively engaged in reabsorbing glucose. The small amount of glucose given, the time when it was given and the fact that there was no glycosuria all make this improbable.

The diodone/inulin clearance ratios are lower than those of adults. This must indicate that at the lowest levels of plasma diodone the tubules excrete diodone less readily than they do in adults but the interpretation of the results in a quantitative sense is very difficult. Thus, if the glomerular filtration rates are lower in infants than they are in adults because the glomerular tufts in infancy

are covered with a layer of cubical cells, then a larger proportion of the diodone in every 100 c.c. of plasma passing through the infant kidney will be presented to the tubules for excretion. For any given level of plasma diodone, therefore, infant tubules will have more diodone to excrete than adult tubules in order to free the plasma of diodone in one passage through the kidney. The amounts of diodone excreted by glomerular filtration in infants may, however, be higher than one would at first sight expect. In the first place the plasma proteins are lower at birth than at maturity, and less diodone will be adhering to them. More of the plasma diodone, therefore, will be free for ultrafiltration. In the second place, some protein is generally to be found in the urine at birth, and this protein presumably comes through the glomeruli with some diodone adsorbed on to it. The diodone/inulin clearance ratios were always reduced by 40–60% when the level of diodone iodine in the plasma was raised to about 30 mg./100 c.c. Figures for these clearances and for diodone $Tm.$ will be discussed in a subsequent communication, together with work on glucose $Tm.$ and the excretion of p-amino-hippurate.

The creatinine clearances in adults, like the diodone clearances, are depressed when the plasma creatinine is raised sufficiently high, but there should be no observable depression so long as the plasma values lie below 15 or 20 mg./100 c.c. It will be observed, however, that the creatinine clearances were of the same order as the inulin clearances. The creatinine/inulin ratios, in fact, varied from 0·68 to 1·32 and were on the whole about 1. There is nothing in these results to suggest that infants' tubules excrete creatinine as those of adults appear to do. It is, of course, possible that the creatinine is being excreted by one part of the tubules and leaking back to the blood in another, but this is pure conjecture. In studying the serial creatinine clearances of these babies it has been noted that in five out of the seven babies the creatinine/inulin clearance ratio tended to rise with successive periods. There is no obvious explanation of this observation which may be fortuitous or due to some technical cause. It is felt, however, that it is wise to place it on record.

The magnitude of the urea clearances and their relationship to the inulin clearances differ in no important respects from the findings of McCance & Young (1941). In every child, however, a large rise or fall in the minute volume of the urine was followed by a change of the urea clearance in the same direction. Gordon, Harrison & McNamara (1942) did not find that the urea clearances varied with the minute volumes, but this is certainly to be expected from all that is known about urea clearances in adults, and about the renal function of very young children.

DISCUSSION

Since the diodone/inulin clearance ratios are lower in infants than they are in adults, and since the creatinine/inulin clearance ratios are about 1·0, it is probable that the tubules at birth have lower excretory powers than they have

later in life. The development of these functions must take place as the children grow, but it is not yet possible to state by what age it is normally complete. The adult capacity to excrete diodone may not be reached for some months, for it is well known clinically that very large doses of diodone may have to be given to young children if the renal tract is to be visualized. Neither diodone nor creatinine are of great significance for the clinician, for diodone is a substance foreign to the body, and the excretion of creatinine has no pathological importance. These observations, however, have considerable physiological interest, for they demonstrate functional immaturities of the tubule cells at, and for some time after, birth.

SUMMARY

In babies aged 2–8 days it was found that:

1. The averaged inulin clearances varied from 16·3 to 44·6 c.c./1·73 sq.m./min.

2. The averaged diodone clearances at low levels of plasma iodine varied from 23·5 to 125 c.c./1·73 sq.m./min. The averaged diodone/inulin clearance ratios varied from 1·39 to 3·37. The average diodone/inulin ratio for all the children taken together was 2·34.

3. The diodone clearances and the diodone/inulin clearance ratios were depressed by raising the plasma diodone.

4. The creatinine clearances were of the same order as the inulin clearances. The average creatinine/inulin clearance ratio for all the children taken together was 0·98.

The above results indicate that soon after birth diodone is excreted by the tubules but not so efficiently as it is in later life, and that creatinine is not excreted by the tubules at this age.

5. The urea clearances were of the same order of magnitude as those found by previous workers in children of this age. They varied, but not closely, with the minute volumes of the urine, and their relationship to the inulin clearances was similar to that found by other workers.

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THE MECHANISM OF THE STING OF THE COMMON NETTLE (*URTICA URENS*)

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It is generally assumed that the reaction of the human skin to the sting of the common nettle (*Urtica Urens*) is brought about by an unknown poison, present in the fluid of the nettle hair, which has the property of releasing histamine (or H-substance) from the epithelial cells. The nettle hair consists of a fine capillary tube calcified at its lower and silicified at its upper end and closed at the tip in the form of a little bulb. This bulb breaks off in a predetermined line when it comes in contact with the skin, leaving exposed a fine needle-like point formed by the upper tapering part of the hair. As a result of the pressure of contact this fine tube penetrates the skin and the compression of the bladder-like base injects the contained fluid into the minute wound. According to Haberlandt (1886) the active poison could be an enzyme, and the mechanism of the nettle sting would then be comparable with that of the bee sting, bee venom being a lecithinase which acts on lecithin forming a lytic substance (lysolecithin) which then in its turn releases histamine (Feldberg & Kellaway, 1937*a*, *b*). Haberlandt certainly excluded the possibility that formic acid is the active substance in nettle hairs, but he performed no experiments on the action of nettle hair extracts on isolated tissue preparations.

Our experiments were started on the assumption that the pharmacologically active substance in the nettle hair acts by the release of histamine from the human skin. This might occur if the substance behaved like an antigen in the antigen-antibody reaction, or if it were an enzyme. However, a different and in some ways simpler explanation was found. The hair fluid itself contains histamine and in a high concentration. It was also found to contain acetyl-choline in an even higher concentration and a third smooth muscle-contracting substance which has not yet been identified. The mechanism of the nettle sting reaction can be explained wholly, or at least in its main features, by the presence of histamine and acetylcholine in the hair fluid; there is no necessity to assume that the 'nettle poison' acts by releasing additional histamine from the human skin. It was further shown that histamine as well as acetylcholine occur not only in the hair fluid but also in the tissue of the leaves.

METHODS

For testing the hair fluid on isolated tissue preparations the hairs were removed with fine forceps from the stalks or the leaves of the plant and either individual hairs were added to the bath containing the smooth muscle preparation, or extracts of a number of hairs were prepared. For this purpose 10–2000 hairs were removed, and were placed in a known volume, usually 1 or 2 c.c. of distilled water or saline solution, and the fluid was shaken for a moment. When extracts were used for injection they were centrifuged and filtered to remove any solid fragments of the hairs. Extracts of leaves were prepared by grinding small pieces in a mortar with a definite volume either of saline solution or of distilled water.

The extracts were tested either on the isolated guinea-pig's or rabbit's intestine preparation suspended in a 10 c.c. bath containing magnesium-free Tyrode's solution, on the eserinized frog's rectus muscle suspended in 5 c.c. Ringer's solution, on the arterial blood pressure of the cat or rabbit anaesthetized with chloralose or urethane respectively, or on the vessels of the external ear of the rabbit perfused with saline solution containing 0·9 g. NaCl, 0·01 g. NaHCO$_3$, 0·01 g. KCl and CaCl$_2$ per litre.

In order to compare the effects of the nettle sting reaction with those of histamine and of acetylcholine a drop of a freshly prepared solution of these substances was placed on the skin of the forearm and the skin was pricked superficially through the drop with a very fine needle. This method has been used by Lewis (1927) and his co-workers.

The effects of extracts were compared with histamine dihydrochloride and with acetylcholine chloride. All values refer, not to the base, but to the salt.

RESULTS

Pharmacological actions of the hair fluid

Acetylcholine

When a single hair is removed from its base with forceps and then placed into a 10 c.c. bath in which is suspended a piece of intestine, either from a guinea-pig or rabbit, a strong contraction occurs and the muscle relaxes again as soon as the bath fluid is replaced by fresh Tyrode's solution. In Fig. 1 are seen such effects of single hairs on the small intestine preparation of the rabbit (*a*) and the guinea-pig (*b*). It appeared unlikely that the effect was due, like the contractions produced by bee venom, to an enzyme action on the intestinal muscle, since the effect was immediate and no desensitization occurred. When the muscle had relaxed the addition of new hairs to the bath again produced contractions. In addition, the active substance in the hair fluid was heat resistant.

The contractions produced by *single* hairs could not be due to histamine since the rabbit's intestine is very insensitive to it. That the contractions were due to acetylcholine was shown by the following experiments.

(1) The addition of 1 or 2 hairs to a 5 c.c. bath containing the eserinized frog rectus muscle caused it to contract (Fig. 1c).

(2) When ten hairs were added to 10 c.c. of Ringer's solution and 1 or 2 c.c. of this solution was injected intravenously into a cat or a rabbit a fall of arterial blood pressure resulted (Fig. 1d).

(3) The smooth-muscle-contracting effects and the depressor actions were abolished by small doses of atropine. In the experiment of Fig. 2 this effect of
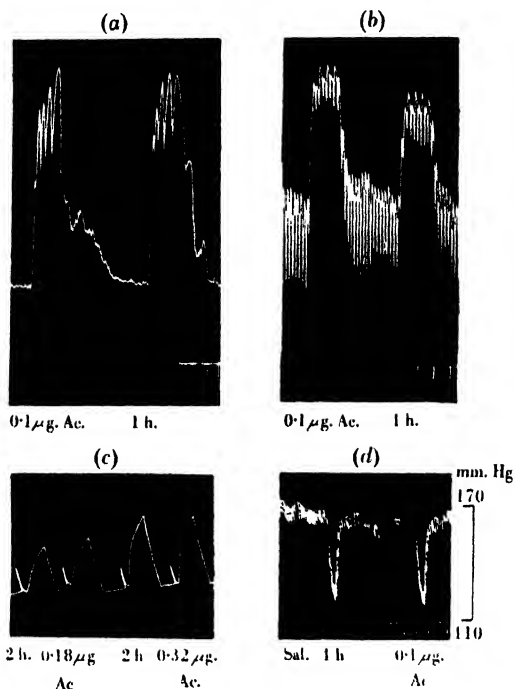
Fig. 1. Comparison of the effect of hairs (h.) from the stalk of the nettle plant with that of acetyl-choline (Ac.) on guinea-pig's intestine (*a*), rabbit's intestine (*b*), eserinized frog rectus muscle (*c*) and on arterial blood pressure of the cat in chloralose anaesthesia (*d*). Hairs and acetylcholine left in intestinal bath for 60 sec. and in frog rectus bath for 90 sec. Sal., injection of 2 c.c. saline solution. Time in (*a*) and (*b*) 30 sec., in (*d*) 10 sec.
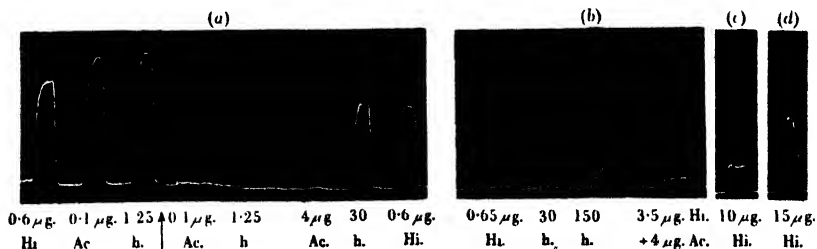
Fig. 2. Contraction of the isolated guinea-pig's jejunum in 10 c.c. bath. Comparison of nettle hair extract (h.) with histamine (Hi.) and acetylcholine (Ac.) before and after atropine and thymoxy-ethyl-diethylamine. From arrow (↑) on till end of experiment atropine 1 in 20 millions and in (*b*), (*c*) and (*d*), in addition, thymoxy-ethyl-diethylamine 1 in 2 millions. Histamine, acetyl-choline and hair extract kept in the bath for 1 min., except the last 2 contractions of (*b*) when kept for 2½ min. (for details see text).

atropine is shown on the contraction produced by the extract from 1·25 hairs on the guinea-pig's intestine.

(4) On the other hand with the anti-histamine substance thymoxy-ethyl-diethylamine it is possible to produce paralysis of the guinea-pig's intestine so that the muscle no longer responds to histamine but continues to respond to acetylcholine and to the hair fluid. The experiment of Fig. 3 illustrates the close parallelism in the reactions to acetylcholine and to hair fluid when the intestinal muscle is treated with thymoxy-ethyl-diethylamine. Before this substance was given, the effect of 0·6 μg. of histamine was stronger than that of 0·2 μg. of acetylcholine and of extract from 2·5 hairs. Thymoxy-ethyl-diethylamine (1 in 2 millions) rendered the muscle insensitive to this dose of histamine, but the acetylcholine as well as the hair fluid had each retained a small fraction of
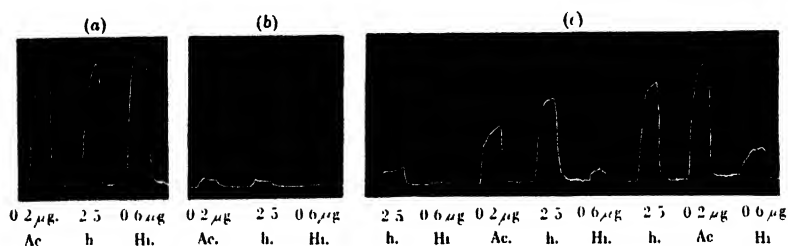
Fig. 3. Contractions of the isolated guinea-pig's jejunum in 10 c.c. bath. Effect on the non-atropinized preparation of thymoxy-ethyl-diethylamine (1 in 2 millions) on the action of histamine (Hi.), acetylcholine (Ac.) and extract from nettle hairs (h.) (left in bath for 1 min.) (a) before, (b) during and (c) after thymoxy-ethyl-diethylamine (for details see text).

its original activity. On washing out the antihistamine substance the muscle regained its sensitivity to acetylcholine and to the hair fluid simultaneously and much earlier than to histamine.

(5) The physical and chemical properties of the active substance in the hairs were identical with those of acetylcholine. To demonstrate these properties extracts of 100 hairs were used. The active substance in these extracts was found to be dialysable, insoluble in ether, sensitive to alkali and resistant to boiling in acid or neutral solution. Like acetylcholine it was inactivated by cholinesterase, but not in the presence of eserine. Dried horse plasma was used as a preparation of cholinesterase.

(6) When an extract was assayed against acetylcholine on the guinea-pig's intestine, the rabbit's intestine and the frog rectus muscle, the values of acetylcholine obtained with all three tests were approximately the same. For instance a solution was prepared from 100 hairs derived from the stalk and assayed in this way. All three tests gave the acetylcholine content of the solution as more than 12 μg. and less than 15 μg. When a further approximation was attempted there was often no absolute agreement, the value obtained by

assay on the guinea-pig's intestine being somewhat higher than those obtained with the assay on the other two tissues. This can be explained by the fact that histamine is also present in the hair fluid (see p. 446). Of the three tissues used for the assay, the guinea-pig's intestine alone is sensitive to small doses of histamine.

It proved possible to determine the acetylcholine content of individual hairs. Since each hair can be tested once only, the values are naturally approximate, but if the sensitivity of a preparation remains relatively constant the values are reliable. This is shown by the fact that the average acetylcholine content of nettle hairs was the same whether obtained from a series of tests on individual hairs or from a single extract prepared from a number of hairs from the same region. For instance, the average value for the 10 hairs tested separately and given in Table 1, row 2, was $0.108\,\mu g.$, the average value determined from an extract prepared of 10 hairs from the same stalk $0.106\,\mu g.$ Corresponding figures from 10 hairs from the leaves were 0.035 and $0.03\,\mu g.$

TABLE 1. Acetylcholine content of individual hairs

Source of hairs	μg. acetylcholine of individual hairs										Av.
Stalk	0·03	0·15	0·14	0·06	0·025	0·1	0·06	0·05	0·01	0·025	0·066
Stalk	0·04	0·025	0·075	0·4	0·065	0·08	0·06	0·018	0·045	0·1	0·108
Leaves, upper surface	0·045	0·04	0·036	0·046	0·034	0·033					0·039
Leaves, lower surface	0·025	0·05	0·037	0·02							0·031

Table 1 shows the acetylcholine content of individual hairs from the same plant. The values in each row are given in the order in which the hairs were removed and assayed. The values in the first row were obtained by the use of the rabbit's intestine, while those in the other rows were obtained with the guinea-pig's intestine. It is evident (*a*) that there are relatively great individual variations in the acetylcholine content ($0.01–0.4\,\mu g.$), even of hairs from adjacent parts of the same plant, and (*b*) that the hairs from the stalk contain, on the average, more acetylcholine than the hairs from the leaves. There are, on the other hand, no significant differences in the acetylcholine content of the hairs which are taken from the upper or lower surface of the leaves.

The individual variations are partly explained by the size of the hairs, the larger hairs usually containing a larger amount of acetylcholine than the smaller hairs, but exceptions to this rule were found. The fact that the hairs from the leaves contain less acetylcholine than those from the stalk is probably due to difference in size.

In Table 2, the average acetylcholine content is given for hairs from different plants, using extracts prepared from 2 to 1500 hairs for the assay. The number of hairs used for each assay and the method of assay employed are indicated

for each experiment. The acetylcholine content of the hairs was of the same order in different plants, but always the hairs from the leaves contained less acetylcholine than those from the stalks. From the figures of Tables 1 and 2 we may conclude that the average acetylcholine content per hair from the stalk is of the order of $0.1\mu g$. and that from the leaves about a third to a half of this value.

TABLE 2. Average acetylcholine content of hairs

Origin of hairs	No. of hairs used for assay	Acetylcholine content in μg. per hair	Test used for assay
Stalk	10	0·106	Guinea-pig's gut
Stalk	100	0·135	Guinea-pig's gut
			Rabbit's gut
			Frog rectus
Stalk	500	0·125	Guinea-pig's gut
Stalk	1000	0·078	Guinea-pig's gut
Stalk	10	0·11	Rabbit's gut
Stalk	2	0·16	Frog rectus
Stalk	4	0·075	Frog rectus
Stalk	100	0·077	Frog rectus
Stalk	1500	0·09	Frog rectus
Stalk	10	0·1	Cat's blood pressure
Leaves	10	0·03	Guinea-pig's gut
Leaves	20	0·035	Frog rectus
Leaves	40	0·053	Frog rectus

$0.2\mu g$ $0.3\mu g$.   (a)   (b)   (c)   (d) $0.3\mu g$.
Hi.   Ac.   Ac.
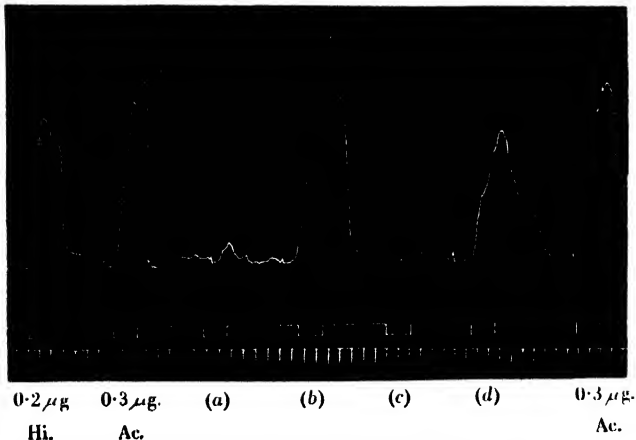
Fig. 4. Contractions of the isolated guinea-pig's intestine in 10 c.c. bath. Immersion for 1 min. of piece of nettle leaf with three hairs. At (a) hairs left intact; (b) hairs broken in bath; (c) hairless leaf; (d) extract from same piece of hairless leaf. Hi. and Ac. = histamine and acetylcholine respectively. Time in 30 sec. (for details see text).

In order to estimate the volume of fluid present in the hairs, 500 hairs from the stalks were collected and weighed before and after drying on the water bath. The fresh weight was 7·0 mg. and the dry weight 2·5 mg. This high proportion of dry material is due to the fact that the hair is partly calcified and partly silicified. If the total fluid content of 500 hairs is 4·5 mg. then the mean

fluid content of each hair is $9\mu^3$. The value agrees well with the value of $7-8\mu^3$ given by Haberlandt (1886). On the basis of these figures, the average concentration of acetylcholine in the hair fluid is about 1 in 100 or stronger.

The acetylcholine present in the hairs is expelled only when the hairs are broken. This is shown in Fig. 4. At a, a piece of leaf containing 3 hairs was placed for 1 min. in the bath containing the guinea-pig's intestine. No contraction resulted. The procedure was repeated at b, with the difference that the hairs were broken off from the leaf in the bath with forceps. At once a strong contraction ensued. The leaf was then removed and the bath fluid replaced with fresh Tyrode's solution. A renewed immersion of the now hairless piece of leaf had no effect (c). For comparison the effects of the addition to the bath of $0.3\mu g$. acetylcholine and of $0.2\mu g$. of histamine are shown.

Histamine

The presence of histamine in the hair fluid was suspected from the fact that an extract prepared by expelling the content of 1000 hairs into 2 c.c. of saline solution gave a small but definite triple response when pricked into the human skin. The extract contained acetylcholine in a concentration of about 1 in 20,000, but the triple response could not have been due to this, since, even in much stronger concentrations, acetylcholine produces no triple response in the human skin* but only a localized vasodilatation. The presence of histamine in the nettle-hair extract would not have become evident in the assay for acetylcholine on the histamine insensitive frog rectus muscle or on the rabbit's intestine which responds to very large doses only. On the other hand, the guinea-pig's jejunum is nearly as sensitive to histamine as it is to acetylcholine, but if the amounts of histamine in the extracts are 10% or less of those of acetylcholine it would not interfere, or only to a slight extent, in the assay. In fact it was found, as mentioned before, that the assay for acetylcholine gave slightly higher values when carried out with the guinea-pig's jejunum instead of with the frog rectus or with the rabbit's gut.

To test for the presence of histamine in the extracts of nettle hairs the acetylcholine could be destroyed with alkali at room temperature. It was not, however, possible to boil the extract in alkaline solution without also inactivating the histamine to a great extent. We found, for instance, that 80% of histamine was inactivated when a solution 1 in 1 million was boiled for 10 min. in $1/30$ N-NaOH. The usual procedure, therefore, was not to destroy the acetylcholine, but to assay the extract on the atropinized guinea-pig's intestine, after a preliminary test had shown that the muscle was atropinized sufficiently to prevent its response to acetylcholine in amounts greater than those contained in the extract.

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* This statement does not apply to the rare cases of allergic hyper-sensitivity to acetylcholine described by Grant, Pearson & Comeau (1935).

The atropinized guinea-pig's gut does not respond to the addition of 1 or 2 hairs, but if extract equivalent to 15–30 hairs is added to the bath a strong contraction ensues. In the experiment of Fig. 2, for instance, equally strong

TABLE 3. Average histamine content of hairs

Origin of hairs	No. of hairs used for assay	Histamine content in μg. per hair
Stalk	1000	0·022
Stalk	500	0·0085
Stalk	1500	0·011
Leaves	1000	0·005

contractions were produced by extract from 1·25 hairs and by 0·1μg. of acetylcholine. In the presence of atropine 1 in 20 millions, however, the muscle did not respond even to 4μg. of acetylcholine; similarly, the extract from 1·25 hairs was now ineffective, but if the equivalent of 30 hairs was added a contraction ensued, although the acetylcholine added in this way was less than 2·5μg. The effect of 30 hairs corresponded to that of 0·65μg. of histamine, i.e. about 0·022μg. of histamine per hair. With this procedure the histamine equivalent of three other extracts was assayed. The results are given in Table 3. From these the concentration of histamine in the hair fluid can be calculated; it is about 1 in 1000 to 1 in 500 or even stronger.

The actual identification of histamine in the hair fluid was rendered difficult on account of the presence of yet another smooth muscle-contracting substance in the hair fluid, which, however, produced effects only if large amounts of extract were used. Usually, interference by this substance could be avoided if the amounts of extract employed were kept as small as possible. If this was done it could be shown that histamine was responsible for the contractions produced on the atropinized guinea-pig's intestine by an extract equivalent to 15–30 hairs. The evidence is based on the following experiments.

(1) The anti-histamine substance, thymoxy-ethyl-diethylamine, affected the contractions produced on the atropinized guinea-pig's gut by extract from 15 to 30 hairs in the same way as it affected equally strong histamine contractions.
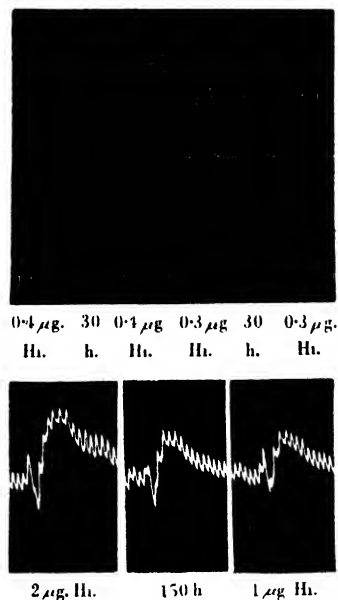
Fig. 5. Assay of nettle hair extract (h.) against histamine (Hi.) on atropinized guinea-pig's intestine (upper tracing) and on arterial blood pressure of atropinized cat in chloralose anaesthesia (lower tracing). Time in upper tracing 30 sec.

In the experiment of Fig. 2, for instance, the contraction caused by 30 hairs or by 0·65µg. of histamine were both abolished in the presence of thymoxy-ethyl-diethylamine 1 in 2 millions.

(2) Extract equivalent to 15–30 hairs had no effect on the isolated atro-pinized rabbit's intestine preparation which responded to very large doses of histamine only (over 50µg.).

(3) Like histamine the extract produced a fall of arterial blood pressure in the atropinized cat. Furthermore, when assayed on this preparation it gave the same histamine value as when assayed on the atropinized guinea-pig's gut. For instance, in the experiment of Fig. 5 an extract from 1500 hairs was assayed on both preparations. The effect of extract from 30 hairs was stronger than that of 0·3µg. and weaker than that of 0·4µg. of histamine on the atropinized gut; on the atropinized cat's blood pressure 5 times the amount of extract was stronger than 1µg. and weaker than 2µg. of histamine.

(4) As mentioned before, the extract of hairs gave a small triple response. When the histamine concentration of such an extract had been assayed on the atropinized guinea-pig's gut and a histamine solution of corresponding strength was pricked into the human skin it produced an indistinguishable reaction.

A third unidentified smooth muscle-contracting substance

The fact that hair fluid contains in addition to acetylcholine and histamine a third smooth muscle-contracting substance became obvious when large amounts of extract were tested on the atropinized rabbit's intestine. For instance, in the experiment of Fig. 6 extract from 170 hairs produced a slowly developing contraction when left in contact with the muscle for 2 min. The histamine and acetylcholine added with the extract would have been not more than 2 and 20µg. of histamine and acetylcholine respectively, but no con-traction occurred when even 30µg. of acetylcholine or 3µg. of histamine were added to the bath, either separately as shown in Fig. 3, or together. In order to produce a contraction comparable to that caused by extract from 170 hairs, over 100µg. of histamine would have to be added. When the hair extract was washed out, the muscle relaxed slowly and when completely relaxed, the renewed addition of extract produced the same effect. The muscle thus did not become insensitive to the hair extract on its repeated administration.

The effect of hair extract on the atropinized rabbit's intestine differed from that of histamine in that it was more resistant to the anti-histamine substance thymoxy-ethyl-diethylamine which, in a concentration of 1 in 2 millions, abolished a contraction produced by the addition of 200µg. of histamine to the bath, but only slightly depressed a similarly strong contraction produced by hair extract. This observation led us to test the effect of the unidentified sub-stance in hair extract also on the atropinized guinea-pig's intestine, when its sensitivity to histamine was depressed by thymoxy-ethyl-diethylamine. In the

experiment of Fig. 2 the atropinized guinea-pig's intestine was treated with this substance (1 in 2 millions) at *b*; now it responded with only a small contraction to 3·5 μg. of histamine. When extract from 150 hairs, containing about this amount of histamine, was added to the bath there occurred at once a small contraction due to histamine, followed by a further slow contraction of greater magnitude, which could not be attributed to the histamine content.

The unidentified substance apparently also contracted the vessels of the perfused rabbit's ear. According to Rothlin (1920) these vessels contract to 0·00001μg. histamine; in our experiments, 0·5–1 μg. (in 0·2 c.c.) were needed to produce vasoconstriction. The amount of extract containing these amounts of histamine would correspond to between 50 and 100 hairs. It was found, however, that extract equivalent to 20 hairs (in 0·2 c.c.) usually caused a

170 h.   30 μg.   3 μg.   100 μg.
                  Ac.   Hi.   Hi.
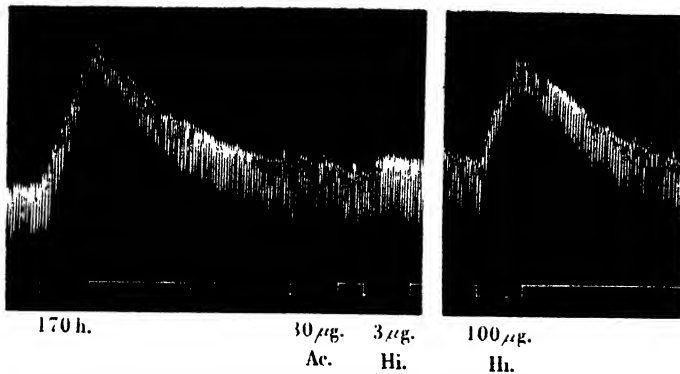
Fig. 6. Contractions of isolated atropinized rabbit's intestine in 10 c.c. bath (atropine 1 in 20 millions). Comparison of the effect of extract from 170 hairs (h.) with 30 μg. of acetylcholine (Ac.) and 3 and 100 μg. of histamine (Hi.) respectively, kept in bath for 2 min.

vasoconstriction which was much more intense and lasted longer than that produced by 1 μg. of histamine. When the vessels had become less sensitive to histamine in the course of the perfusion and reacted only to 10 μg. histamine or more, they still contracted to extract equivalent to 100 hairs. It is probable, although not certain, that this smooth muscle-contracting effect is due to the same substance which is responsible for the contraction produced on the atropinized rabbit's intestine.

The arterial blood pressure of the atropinized rabbit under urethane anaesthesia is very insensitive to histamine. However, the intravenous injection of extract equivalent to 200–500 hairs, containing between 2 and 10 μg. histamine, caused an immediate sharp and transient fall in arterial blood pressure. A similar effect can sometimes be produced in this preparation by much larger doses of histamine than those injected with the extract, and it is then the result of constriction of the pulmonary arteries. We have not determined whether the

depressor action of the extract was to be explained on similar lines. In that case it would also represent an effect on smooth muscles and probably be due to the same substance which contracts the atropinized rabbit's intestine.

The active substance responsible for the smooth muscle-contraction is inactivated to a great extent by boiling in alkaline solution. In this way it resembles histamine. Unlike histamine, however, it is soluble in ether. It is certainly not potassium, ephedrin or tyramine. The effects are also not due to the presence of formic acid. It is true that the hairs, when crushed on litmus paper, give an acid reaction which may be due to traces of this acid, but the pH is not very low. Crushing the hairs on congo red paper did not colour it
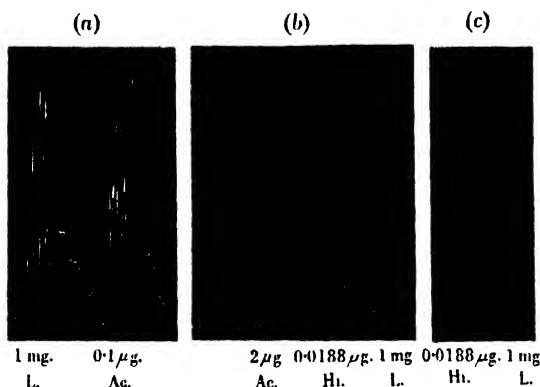
Fig. 7. Contractions of the isolated guinea-pig's intestine in 10 c.c. bath. Comparison of the effect of extract from 1 mg. nettle leaf (L.) with acetylcholine (Ac.) and histamine (Hi.); all left in bath for 1 min. Between (a) and (b) till end of experiment atropine 1 in 20 millions; at (c) in addition thymoxy-ethyl-diethylamine 1 in 2 millions.

brown or even blue. In addition, a watery extract prepared from 1000 hairs in 1 c.c. distilled water was neutral in reaction. Formic acid, moreover, even at a 1 % concentration or more did not imitate any of the effects of the extract.

Acetylcholine and histamine content of leaves

Acetylcholine and histamine occur not only in the hairs, but are also present in the leaves of the nettle plant. In order to demonstrate their presence, the leaves have to be crushed. Grinding them in Ringer's solution is sufficient for this purpose. In the experiment of Fig. 4 it was shown that a piece of leaf introduced into a 10 c.c. bath containing the guinea-pig's intestine caused no contraction (at c). When the same piece of leaf, however, was ground in saline solution and the fluid then added to the bath, a contraction ensued (at d). The contraction is due to some extent to acetylcholine, but mainly to histamine. Unlike the hairs, the leaves contain the two substances in about equal concentrations or sometimes contain more histamine than acetylcholine. Atropine, therefore, only reduces but does not abolish the contraction of the gut muscle

caused by extracts from the leaves. This is illustrated in the experiment of Fig. 7 in which the effect of 1 mg. leaf extract was tested before and after atropine on the guinea-pig's intestine and compared with those of acetylcholine and histamine. It is thus not possible to determine the acetylcholine content of leaf extract by assaying it on the non-atropinized guinea-pig's intestine. The assay is best carried out on the eserinized frog rectus muscle. Two leaf extracts assayed in this way gave $0\cdot018$ and $0\cdot027\mu g.$ of acetylcholine per mg. leaf tissue. The first of these extracts was also assayed for histamine on the atropinized guinea-pig's intestine; it contained $0\cdot019\mu g./mg.$ A third extract contained $0\cdot05\mu g./mg.$

The identification with histamine was supported by showing that thymoxy-ethyl-diethylamine abolished the contraction of leaf extract on the atropinized guinea-pig's gut (see Fig. 7c) and that the atropinized rabbit's intestine which only responded to $100\mu g.$ did not contract to the addition of extract from 10 mg. leaf tissue. The concentration of both these substances in the leaves is surprisingly high, but much lower than that found in the hair fluid. The acetylcholine and histamine could not have originated from the bottom parts of the hairs since only hairless parts were used in the preparation of the extracts. No experiments have yet been done concerning the presence in the leaf tissue of the third unknown smooth muscle-contracting substance which was found in the hair fluid.

Histamine and acetylcholine on the human skin

Solutions of both substances were pricked into the human skin in order to find out whether histamine and acetylcholine can imitate the vascular reactions and the sensations produced by the sting of the nettle hair.

Acetylcholine even in a concentration as high as 1 in 50 produces no wheal or occasionally a very small one only. The acetylcholine prick may be followed by an indefinite and weak sensation of pain. Thus, neither the vascular reactions nor the sensations produced by acetylcholine resemble those of the sting of the nettle hair.

Histamine, 1 in 2000 to 1 in 1000, causes the well-known triple response which is indistinguishable from that produced by the sting of the nettle hair. The vascular reaction of the nettle sting, therefore, is accounted for wholly by the histamine present in the hair fluid. There is, however, the following difference. Histamine, applied in this way, is associated with strong itching which begins about 40–50 sec. after application. The sting of the nettle hair also causes itching, but the first sensation is that of intense burning pain, and in the German language the plant is actually called burning nettle ('Brenn-nessel'). Histamine only occasionally gives a burning sensation which even then is weak. Usually there is some slight sensation of indefinite character before the itching starts.

The sensation, however, becomes altered when histamine, 1 in 2000 or 1 in 1000, is pricked into the skin together with acetylcholine, 1 in 50 or 1 in 100. After 15–25 sec. a strong burning sensation is felt, followed after about another 30 sec. by itching, which outlasts the burning sensation. Several persons were pricked on one arm with a solution containing histamine only and on the other with one containing acetylcholine as well. They were not told which solution contained both substances and this fact was also unknown to the person who performed the experiment. In four out of five cases they were able to distinguish sharply between the two sensations and often stated definitely that the sensation produced by histamine and acetylcholine resembled that produced by the natural nettle sting. The following is a protocol of a typical experiment. On a human subject a drop of histamine, 1 in 2000, was placed on the flexor surface of the right forearm and a drop of histamine, 1 in 2000, plus acetylcholine, 1 in 60, was placed in a corresponding position on the left forearm. The skin beneath each drop was pricked superficially three times with a needle.

Protocol

Time after prick in sec.	Sensations felt after histamine	Sensations felt after histamine + acetylcholine
20	Faint indefinite sensation	Burning
40	Faint pricking	Burning more pronounced, like a nettle sting
60	Itching starts but no burning	Violent itching starts
90	Itching	—
120	Itching	Itching without any more burning, like the sensation on the other arm

In order to obtain these sharp differences in the sensations felt, fine needles have to be used for pricking and the room has to be kept warm. If the person feels cold it becomes difficult to feel pronounced itching when histamine alone is pricked into the skin. When the skin is pricked with single hairs the burning sensation sometimes also fails to appear, but it is often stronger than that produced by histamine plus acetylcholine. It was thought that formic acid, supposed to be present in the hair fluid, might contribute to the burning sensation produced by the sting of the nettle. However, no evidence could be obtained for this assumption. The addition of 1 % formic acid to the solutions did not alter the sensations produced by histamine with and without acetylcholine. A 1 % solution of formic acid is certainly much more acid than the reaction of the hair fluid. In order to obtain sensations with formic acid it must be pricked into the skin in a concentration stronger than 5 %. If it is 20 % or stronger intense pain is felt, even when the strong acid is applied to the intact skin.

DISCUSSION

The view hitherto held was that the vascular reactions on the human skin caused by the sting of the nettle hair are brought about by the release of histamine or of a histamine-like substance from the epithelial cells of the skin. As a result of the experiments here reported this view can no longer be accepted. Histamine itself is present in the hair fluid and in a concentration sufficient to account for the characteristic triple response. The similarity between the reactions produced by histamine and those of the nettle hair thus finds a simple explanation. In fact, whenever stings of other plants or bites and stings of parasites and insects produce histamine-like reactions the possibility must be considered that histamine is introduced into the skin and not necessarily released from it by an unknown poison.

In addition to histamine the hair fluid contains acetylcholine, often in an extremely high concentration, sometimes stronger than 1 in 100. The absence of any cholinesterase in the hair fluid made it easy to detect and to determine quantitatively the content of acetylcholine in single hairs. It seemed at first difficult to attribute any function to this strong solution of acetylcholine for the skin reactions produced by the sting of the nettle hair. When such a solution of acetylcholine is pricked into the skin the vascular reactions as well as the sensations produced are negligible. The function of the acetylcholine, however, becomes clear when it is pricked into the skin together with histamine. In this case a burning sensation is produced, which histamine alone cannot imitate. Histamine alone causes itching, which may be violent, but in the presence of a strong concentration of acetylcholine a burning sensation is caused which precedes the itching. Therefore the characteristic sensation produced by the sting of the nettle hair is a result of a combined action of both the histamine and the acetylcholine in the hair fluid.

The sensations produced by strong solutions of acetylcholine plus histamine are of interest in connexion with the known actions of acetylcholine on sensory nerve endings. Recently Gray (1947) has produced evidence, in animal experiments, that acetylcholine in a concentration of 1 in 10,000 initiates nerve impulses from sensory endings in the skin and in the mesentery. Of more interest in relation to the effect here described is the sensation elicited on arterial injection of acetylcholine in man. With a 2 % solution of acetylcholine an excruciating 'burning' pain is felt for a few seconds along the course of the artery distal from the point of injection (Ellis & Weiss, 1932; Lanari, 1936; Harvey, Lilienthal & Talbot, 1941). This concentration of acetylcholine is not much stronger than that found in the nettle hair fluid. But, as we have seen, a 2 % solution of acetylcholine pricked into the skin produces no definite sensation. When applied in this way the additional presence of histamine is necessary to give burning pain. The question thus arises whether we are

dealing with the same effect in both cases, when acetylcholine is injected arterially and when it is pricked into the skin together with histamine. If so, why is histamine required in this last condition? The histamine might protect the acetylcholine, or the changes in vascular permeability produced by histamine may be the determining factor, or it may be that the sensory endings become sensitized to acetylcholine when under the influence of histamine. No experiments have been performed to decide this question.

While in normal persons the effect of acetylcholine in the hair fluid of the nettle appears to be limited by its action on sensory nerve endings, it may play an additional role in persons allergic to acetylcholine. Grant *et al.* (1935) have described cases in whom urticaria is provoked by emotion, exercise and warming the body. It is assumed that the abnormality in these cases lies in the fact that acetylcholine, like an antigen, acts as an injurious agent, causing the liberation of histamine or H-substance. The acetylcholine would be released in the skin from the endings of cholinergic nerves. This theory is based on the fact that in these persons choline derivatives and other parasympathomimetic drugs produce the triple response. It would be interesting to know if these persons show a particularly strong reaction when stung by the nettle hair; more pronounced vascular reactions could be expected because in these cases histamine is not only pricked into the skin with the nettle hair fluid, but would be released also from the epithelial cells by the acetylcholine.

No function could be attributed to the third unidentified smooth muscle-contracting substance found in the hair fluid. It is certainly not necessary to explain the vascular reactions produced in the human skin, which are fully accounted for by histamine. There is, however, the possibility that the sensations produced by the nettle sting are not fully explained by the presence of histamine and acetylcholine in the hair fluid. Qualitatively the sensation produced by pricking the two substances into the skin resembles that of the nettle sting, but the intensity of the burning pain is sometimes definitely greater with the nettle than with histamine plus acetylcholine. If the third unidentified substance should prove to have an action on sensory nerve endings, it may act by intensifying the sensations produced by acetylcholine and histamine. On the other hand, it has to be realized that a needle prick through a solution containing histamine and acetylcholine is a crude imitation only of the highly specialized injection mechanism by which the hair expels its fluid into the skin, and this difference may explain the stronger sensation sometimes felt with the nettle sting.

In considering the action of the unidentified muscle-contracting substance in the nettle hair fluid, the possibility has to be kept in mind that although it may not have obvious effects in man it may produce these in animals stung by the nettle hair.

No experiments have yet been done concerning the problem of the origin of histamine and acetylcholine in the hair fluid. The fact that these substances occur in the leaf tissue of the plant, although in weaker concentration, may indicate that they are formed here and transported into the hairs and there concentrated. Both substances, however, may be present in other parts of the nettle plant such as the roots, and as long as our knowledge of their distribution in the plant is incomplete the problem of where and how they are formed cannot be fruitfully discussed.

SUMMARY

1. The fluid in the nettle hair contains at least three pharmacologically active substances: histamine, acetylcholine and an unidentified smooth muscle-contracting substance. The identification of histamine and acetylcholine is based mainly on pharmacological reactions and on some physico-chemical properties.

2. Histamine is present in the hair fluid in a concentration of about 1 in 500 to 1 in 1000 and is responsible for the triple response and the itching sensation produced by the sting of the nettle hair on the human skin.

3. Acetylcholine is present in the hair fluid in a concentration of about 1 in 100 or stronger. The absence of cholinesterase in this fluid made it easy to determine the acetylcholine content of individual hairs. The acetylcholine is responsible for producing the burning sensation of the sting of the nettle hair. It was found that acetylcholine 1 in 100 pricked into the skin caused no definite sensations, but produced burning pain when introduced together with histamine.

4. Histamine and acetylcholine occur also in the leaf tissue of the nettle plant, but in weaker concentrations than in the hairs. The possibility exists that these substances are formed in the leaves, transported to the hairs and there concentrated.

5. No function could yet be attributed to the unidentified smooth muscle-contracting substance present in the fluid of the nettle hair.

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SOME FACTORS AFFECTING THE
ACIDITY OF URINE IN MAN

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The recent failure to find any satisfactory explanation of the increase in urine acidity resulting from ingestion of alcohol (Eggleton, 1946) suggested the necessity for an investigation into possible different types of urine acidity. In the experiments with alcohol, the usual criterion of urine acidity, namely changes in hydrogen-ion concentration, was adopted. In such a buffered solution as urine, however, it is apparent that an increase in hydrogen-ion concentration need not necessarily be due entirely to an increase in excretion of acidic ions: a reduction in the overall buffering power of the solution would enable an unchanged excretion of acidic ions to produce a shift in hydrogen-ion concentration.

Hitherto, little attention has been paid to changes in the total buffering capacity of the urine under various physiological conditions, interest being centred rather on the 'titratable acidity' value. This value is obtained by titration of the sample with standard alkali to about pH 8 (phenolphthalein first pink) and represents the buffering power of the urine over the range pH 8 to the pH at which it was secreted. It is found, naturally enough, to vary directly with the hydrogen-ion concentration, i.e. the lower the pH of the urine sample, the higher its 'titratable acidity' value. It gives no indication of the total buffering capacity of any sample over the whole physiological range pH 4·8–8·0 except for those samples secreted at pH 4·8.

Approach from a completely different angle, that of determination of changes in output of phosphate, suggests that an increase in hydrogen-ion concentration of the urine *may* in some circumstances be associated with an increase in total buffering power. Thus Haldane (1921) noted an increase in phosphate excretion following ingestion of ammonium chloride in man, a result later confirmed by Loeb, Atchley, Richards, Benedict & Driscoll (1932), while Gamble, Ross & Tisdall (1923) observed a similar result after fasting, the increase in phosphate output being greater than that accounted for by wastage of the muscles. Under both sets of conditions, ammonium chloride acidosis and fasting, the prime need of the body is to rid itself of excess acidic ions: by

simultaneously increasing the buffering power of the urine, this end can be achieved without increasing the hydrogen-ion concentration beyond the physiological limits to which the kidney is accustomed.

The results to be presented are concerned with experiments designed to test the possible existence of two distinct types of urine acidity on the lines suggested above.

METHODS

The general procedure used in previous investigations was adhered to, with only slight modifications. The meal previous to the experiment, either breakfast or lunch, was omitted, whether the experimental substance was to be ingested or injected, and a glass of water was usually taken 2–2½ hr. before the experiment began. At least one urine sample was collected during the last 30–40 min. of this resting period, and five or six samples during the 2½–3 hr. following the experimental injection or ingestion. A large number of experiments were performed on one subject and these were repeated, whenever feasible, on groups of other subjects.

In addition to determinations of pH and ammonia concentration (formol titration method) as detailed elsewhere (Eggleton, 1946), the buffering power of the urine was determined. To the 'titratable acidity' value (urine titrated to pH 8·0) was added what might reasonably be called the 'titratable alkalinity' value (urine titrated to pH 4·8, using methyl red-methylene blue indicator), the sum of the two representing the buffering of the urine over the physiological range pH 4·8–8·0. Neither titration is extremely accurate, owing partly to the yellow pigmentation of the urine. This inaccuracy was partially counteracted, and reasonably large titration values obtained even on dilute urine samples, by the use of volumes varying from 5·0–50 c.c. according to the rate of urine flow, the smaller samples being diluted to 50 c.c. in each case. In a few experiments determinations were also made of phosphate output.

RESULTS

The results obtained by Haldane and his successors in regard to the action of acidifying agents such as ammonium chloride were fully confirmed. Two experiments on one subject and one experiment on a second subject were made with ammonium chloride, and one experiment on each of three subjects with ammonium sulphate. In all six a result similar to that shown in Fig. 1 was obtained. Since these substances can be ingested only in dilute solution without inducing vomiting, and since it was desirable, for a reason to be explained later, that they should be taken with a minimum amount of water, they were ingested in the form of enteric-coated capsules. It is clear that, under such conditions of acidosis, a fall in urine pH is accompanied by a pronounced increase in output of buffering substances, of which phosphate accounts for 50–70%. An attempt was made at a later date to give an intravenous injection of hypertonic ammonium sulphate (20% solution) but proved fruitless: nervous symptoms (incipient black-out) and thrombosis occurred after the first c.c. had been injected.

Under all other conditions in which a change in urine pH was observed, a relationship, the exact converse of that shown in Fig. 1, was seen to exist. In Fig. 2, for example, is shown the effect of an intravenous injection of hypertonic sucrose. As the urine acidity increases, the buffer output falls. This experiment was not attempted on any further subjects since it induced
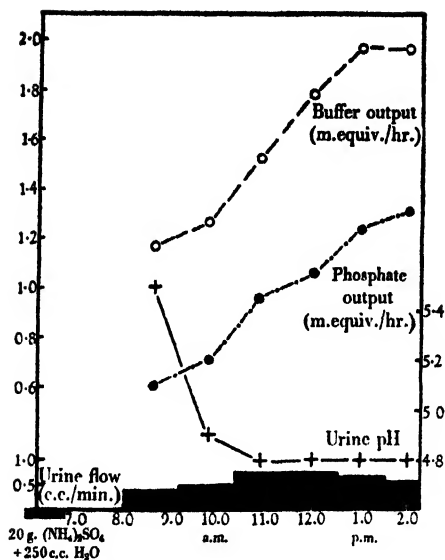
Fig. 1. The effect of ingestion of 20 g. ammonium sulphate (40 enteric-coated capsules) on urine pH, output of phosphate and total buffers.
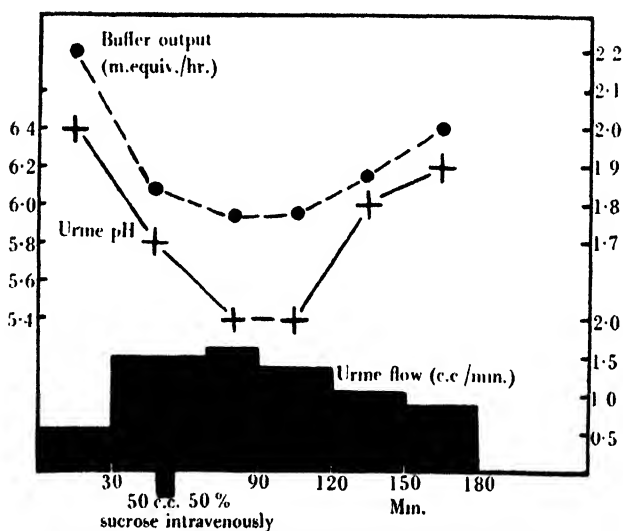
Fig. 2. The effect of intravenous injection of concentrated sucrose solution on urine pH and buffer output.

thrombosis. Hypertonic sodium sulphate, however, was found to produce no ill effects and the average results of an intravenous injection of this substance into four subjects is shown in Fig. 3. Again, the buffer output falls as the urine acidity increases. In a fifth subject the pH fell from 5·6 to 4·8 following the injection, but began to rise again after 30–40 min., and the result, therefore, was not included with those of the remaining four who each individually showed the more prolonged fall seen in Fig. 3. In this figure also is shown the
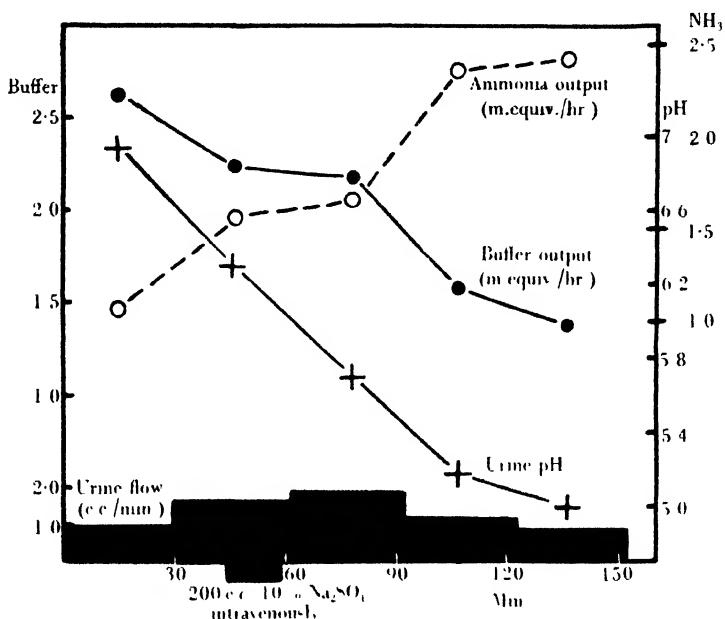
Fig. 3. The effect of intravenous injection of hypertonic sodium sulphate solution on urine pH, buffer output and ammonia output. Average results from four subjects.

inverse relationship which is regularly observed between the outputs of buffer substances and ammonia, a result confirming that previously established between urine pH and ammonia output in alcohol diuresis (Eggleton, 1946).

In contrast with the increase in urine acidity and decrease in buffer output induced by intravenous injection of hypertonic solutions of sucrose or sodium sulphate are the effects, shown in Fig. 4, of the administration by mouth of hypertonic urea solution (the average results of five subjects), but again it is seen that urine pH and buffer output run hand in hand. In one subject an attempt was made to give a rapid intravenous injection of 20% solution of urea; this procedure, however, was not only followed by thrombosis but was so painful that the somewhat erratic course of both urine pH and buffer output which resulted was discounted as being probably connected with the initial nervous disturbance: nor was the experiment repeated on any other subjects.

Ingestion of hypertonic solutions does not necessarily lead to the results shown in Fig. 4. On one occasion, administration of 200 g. glucose in 200 c.c. solution resulted in a fall of urine pH from 6·6 to 4·8, which persisted for the following 2 hr., during most of which time glycosuria was present and the rate of urine flow never greater than 0·63 c.c./min. The experiment was performed with another end in view and buffer output was not determined; the results, therefore, do not merit more than passing mention.

The relationship between buffer output and urine acidity demonstrated in the preceding figures has been observed in some thirty to forty subjects under a variety of conditions, including the naturally occurring variations in acidity
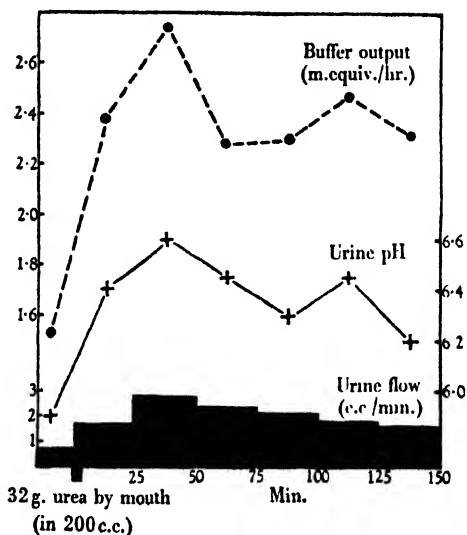
Fig. 4. The effect of hypertonic urea solution taken by mouth on urine pH and buffer output. Average results from five subjects.

encountered during the course of the day. Two factors may, however, mask this relationship at times. Ingestion of tea or coffee results in a disproportionately great output of buffer substances in relation to the pH, but this factor has been eliminated in the series of experiments under consideration. The second factor is that of diuresis itself. A hint of this is to be seen in Figs. 3 and 4 and is shown in pronounced form in Fig. 5. These results again are the average from four subjects who on one occasion took water alone and on another day water, immediately preceded by subcutaneous injection of 1 unit 'pituitrin'. Considerable variations in urine acidity occurred in the different subjects, but in each individual the curve of buffer output followed fairly closely that of urine pH when the rate of urine flow was low. In the water diuresis, however, the large increase in rate of flow, accompanied by a slight fall in urine pH, induced a large increase in buffer output.

This 'flushing-out' effect on buffer substances at high rates of urine flow is sufficiently great to mask the increased acidity following injection of hypertonic sodium sulphate solutions under certain conditions. Two experiments were performed with a different technique from that used on earlier occasions, in an effort to reduce the lag in time between collection of the resting urine sample and injection of the solution; also, the solution was more concentrated (100 c.c. of 20% sodium sulphate) and given in a shorter time in the hope that a more dramatic change in urine acidity might be observed. But these precautions defeated their own ends. In one subject the rate of urine flow rose to
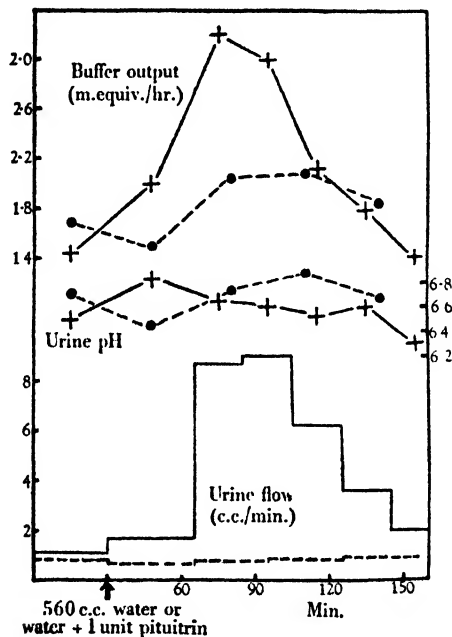
Fig. 5. The effect of rate of urine flow on buffer output. Average results from four subjects +— + 560 c.c. water; ● - - - ● 560 c.c. water preceded by 1 unit 'pituitrin' subcutaneously.

7 c.c./min. and in the other to 4·7 c.c./min. following the injection, and in both subjects this relatively large flow was accompanied by an increase in buffer output and no detectable change in urine pH (7·25 and 7·1 respectively in the two subjects). Incidentally, both subjects noted a sensation of thirst before the end of the injection and both developed a severe headache. The latter symptom had also been observed previously in a subject (probably a mild case of diabetes insipidus) in whom both alcohol and water produced a rapid shift in body water, as indicated by an unusually large and speedy diuresis.

Ammonia excretion. Earlier results (Eggleton, 1946) indicated that the output of ammonia is fairly closely correlated with urine pH, the ammonia rising

as the pH falls: and that, although this ammonia output is affected to some
extent by varying rate of urine flow, no definite relationship exists between
ammonia concentration and urine pH. Those conclusions were drawn from
experiments on one individual, and it has been deemed advisable to extend the
observations by experiments on a group of other subjects and in reference to
urine acidity induced by a number of different agencies.

The results already presented in Fig. 3 show the relationship between
ammonia output and urine pH in a group of four subjects after injection of
sodium sulphate. Ammonia concentration can readily be calculated from the
data given in the figure, and shows no consistent relationship with the pH;
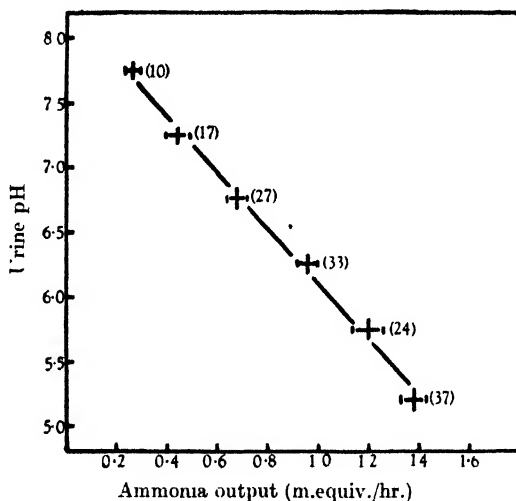
Fig. 6. The relationship between ammonia output and urine pH in one subject, under a variety of
conditions. Average values are given for each half unit of pH. The numbers in brackets
indicate the number of observations included in each average: the distance between the small
vertical lines the standard error of each average. Rate of urine flow varied fifty-fold, from
0·25 to 11·0 c.c./min. in different experiments.

during the stage of increased rate of urine flow, the ammonia concentration
falls as the pH falls (from 21 to 15 m.equiv./l.) and during the later stage of
diminishing rate of flow, the concentration rises considerably (from 33·5 to
46 m.equiv./l.) as the pH changes only from 5·15 to 5·0.

In thirteen out of thirty subjects on whom at least two experiments have
been performed, this relationship between ammonia output and urine pH has
been observed. In regard to the remaining seventeen subjects, no such definite
conclusion could be drawn. Considerable variation was noted among the whole
group of subjects in the extent to which rate of urine flow affected the ammonia
output, and only a more intensive study of each subject over a wide range of
pH and rate of flow could decide which in each is the more fundamental
relationship.

In the subject, for whom data have already been presented from experiments with water and alcohol diuresis (Eggleton, 1946), it has been found that the relation between ammonia output and urine pH remains unaffected when changes in pH are induced by ingestion of urea, or injection of urea, sucrose or sulphate. This relationship, obtained from a large number of experiments of different types, is shown in Fig. 6 in the form of average values for each half unit of pH. The values for ammonia were all obtained by use of the formol titration method, so that the curve for true ammonia would lie about 0·2 m.equiv./hr. to the left of that given.

The results of the experiments in which acidifying agents were ingested have not been included in the averages shown in Fig. 6, for under those conditions the ammonia output is greatly in excess of the values found under other conditions of urine acidity. In the experiment illustrated in Fig. 1, for example, the ammonia output reached a value of over 3 m.equiv./hr.

DISCUSSION

The main point of interest in the results presented is the practical one—that increased urine acidity (hydrogen-ion concentration) may in some circumstances be associated with an increase, and in other circumstances with a decrease in output of buffer substances. The former of these two conditions has been recognized by many workers, but attention has not previously been called to the latter, which appears to be of more general occurrence.

This differentiation between two distinct types of urine acidity argues against Briggs's theory (1934) that the acidifying action of sodium sulphate is due to preferential excretion of the foreign sulphate ion. If that were the case, the increased acidity should be accompanied by an increase in buffer output as with ammonium sulphate, but the reverse is observed.

The fact that sodium sulphate and sucrose, both slowly diffusing substances, lead to an increased acidity of the urine whereas rapidly diffusing urea has no such action suggests that the acidity may be connected with dehydration of the tissues in general. It is tempting to speculate that the post-pituitary antidiuretic hormone may itself be concerned, though the experiments reported here provide no direct evidence on the point. The relatively small diuresis resulting from injection of sodium sulphate or sucrose (although both should be strong osmotic diuretics) as compared with the larger diuresis after ingestion of urea suggests that secretion of the anti-diuretic hormone has been stimulated by the former. This interpretation is fully in accord with Verney's results (1946) on the unanaesthetized dog; intra-carotid injection of hypertonic urea was found to be without effect on a water diuresis whereas hypertonic sucrose, glucose or NaCl produced immediate inhibition. The result of injections of 'pituitrin' might also be cited as additional evidence in favour of this interpretation. The average results of four subjects, given in Fig. 5, show no large

variations, either in rate of urine flow or in urine acidity, owing to individual variations in the degree of response to water plus 'pituitrin', but in each subject the urine pH followed closely the changes in rate of flow.

If this tentative hypothesis be accepted at the moment for lack of a better one, it should be stressed that the acidifying action of 'pituitrin' cannot override an alkalosis of the body. In two subjects to whom 10 g. potassium citrate had been administered, the resulting diuresis was partially counteracted by subcutaneous injection of 1 unit of 'pituitrin'. Under these conditions the diminished rate of urine flow was accompanied by a decrease in acidity: the pH, which had fallen to 7·5 at the height of diuresis, returned to pH 8·0 after the 'pituitrin' injection.

Any attempt at a theoretical consideration of the results from the *renal* point of view at first seemed impossible, since the excretion of individual buffer substances, and carbon dioxide partial pressures, had not been determined. Some further analysis can, however, be made and 1 am indebted to Dr L. E. Bayliss for surveying the results and presenting the following interpretation.

The rate of acid or alkali excretion may be readily calculated from the data in this paper if some assumptions are made as to the initial hydrogen-ion concentration of the glomerular fluid, and as to the changes in hydrogen-ion concentration that would result from the re-absorption of water alone. It is reasonable to suppose that the glomerular fluid has a pH of about 7·4, and that the increase in ionic strength, and reduced activity of the water, during elaboration of the urine will not change this by more than 0·1 pH, but this requires further investigation. The amount of buffer substances excreted, as given in this paper, is measured as the amount of acid required to change the pH of the urine from 4·8 to 8·0, i.e. by 3·2 pH units. As a first approximation, therefore, the amount of acid that must have been added by the tubules, or alkali removed, to bring the urine to any observed value of pH is given by

$$(7\cdot4-\mathrm{pH})\times\frac{(\text{buffer output})}{3\cdot2}$$

(it is assumed that the titration curve of urine is a straight line, which is justifiable over the range of pH considered). It may be remarked in parenthesis that, if this viewpoint be adopted, the rate of acid excretion can be directly measured by titrating the urine to pH 7·4, the titre then giving the acid output, and this, divided by (7·4 *minus* pH of urine as excreted) giving the buffer value.

Calculation of the rate of acid excretion by this method leads to the following conclusions:

(1) Administration of ammonium sulphate led to a considerable increase in the acid output (approximately three-fold), as is obvious from the simultaneous rise in buffer output and fall in pH; this increase was maintained after the diuresis had subsided.

(2) Administration of sucrose or sodium sulphate also led to an increase in acid output (by 50–100%); this acid output fell as the diuresis subsided, but less rapidly. The two types of response to administration of sodium sulphate in respect to changes in pH, the one shown in Fig. 3 (four subjects) and the other in a fifth subject mentioned in the text, had the same type of response in respect to acid output; it is the excretion of buffer substances that behaved differently.

(3) Administration of urea led to no appreciable change in the output of acid, the fluctuations in pH being due entirely to fluctuations in buffer output.

(4) During simultaneous administration of water and pituitary extract, there were small and irregular changes in acid output, which were associated with *inverse* changes in the rate of urine flow. This association broke down on the onset of water diuresis, and in one subject in which the urine went alkaline to pH 7·2.

SUMMARY

1. The increased acidity of the urine after ingestion of ammonium sulphate or chloride is accompanied by an increase in buffer output (titration range 4·8–8·0), of which phosphate accounts for about two-thirds (Fig. 1).

2. Intravenous injection of hypertonic sucrose or sodium sulphate solutions also results in increased acidity of the urine, but this is accompanied by a decrease in buffer output (Figs. 2 and 3).

3. The more pronounced diuresis resulting from ingestion of hypertonic urea solution is accompanied by a decrease in urine acidity, with increase in buffer output (Fig. 4).

4. This inverse relationship between urine acidity (hydrogen-ion concentration) and buffer output may be masked at high rates of urine flow by a 'flushing-out' effect on buffer substances (Fig. 5).

5. Evidence is adduced in favour of the tentative hypothesis that increase in urine acidity, accompanied by decrease in buffer output, may be connected with secretion of anti-diuretic hormone by the post-pituitary.

6. A direct relationship is found between urine acidity and ammonia output under all conditions (Fig. 6).

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VAGAL NERVE FIBRE ACTIVITY FOLLOWING MULTIPLE PULMONARY EMBOLISM

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A striking increase in the rate of breathing of goats commonly follows the intravenous injection of a suspension of potato starch, the grains of which lodge in the small vessels of the lung (Dunn, 1919). This respiratory acceleration is absent if the vagi have been divided, and Christie (1938) suggested that it might be caused by an increase in the activity of the fibres that respond to pulmonary inflation. The experiments described in this paper were designed to discover, first, whether an increased discharge of vagal stretch receptors did in fact follow the injection of potato starch; and secondly, whether the discharge of any other group of vagal afferent fibres was modified.

METHODS

Eight cats and one rabbit, prepared as indicated in Table 1, were used for the first series of experiments. The animals were artificially ventilated and were placed in a warm moist chamber. The cervical vagus was cut across and its peripheral end was dissected until only one active fibre remained. The second series of experiments was conducted on three spontaneously breathing decerebrate cats and one spontaneously breathing anaesthetized rabbit. The animals were placed in an airtight box maintained at constant temperature and fitted with a tracheal tube and venous cannula leading to the outside. The changes in chest volume were registered kymographically by a volume recorder connected with the inside of the box. The connective tissue sheath of one vagus was removed and strands dissected from the side of the nerve were placed on the recording electrodes.

A differential amplifier and cathode-ray tube of conventional design were employed. Arterial and venous pressures were recorded optically by a liquid filled system.

Starch, prepared by filtering a suspension of minced potato through four layers of surgical gauze, was mixed with 20 times its volume of 0·9 % NaCl and was injected into a femoral or jugular cannula.

RESULTS

The vagal afferent fibres encountered most frequently in this work were those responding to inflation of the lungs by discharging a steady series of action potentials. In the first series of experiments the peak frequencies of discharge

of these stretch endings were measured in animals artificially ventilated by constant volumes of air, and were found to be sensibly constant during control periods. Table 1 shows the results of measurements of peak frequencies before and after injections of potato-starch suspensions. No consistent changes were detected, and it is clear that the small random variations observed are insignificant.

TABLE 1. The effect of starch embolism on the activity of vagal stretch receptors

| Preparation | Dose of starch (ml.) | Peak frequency | | Difference |
		Before	After	
Cat-nembutal	10	87·7 ± 4·6 (7)	84·2 ± 0·83 (3)	− 3·5
Do.	10	53·0 ± 2·6 (3)	65·2 ± 64·3 (3)	+ 12·2
Cat-chloralose	20	122·6 ± 4·3 (6)	115·9 ± 9·7 (9)	− 6·7
Cat-decapitate	15	99 (1)	106 (1)	+ 7
Do.	5	152 (1)	169 (1)	+ 17
Do.	5	123 (1)	100 (1)	− 23
Do.	5	98·3 (2)	89·5 ± 6·7 (4)	− 9·3
Cat-decerebrate	15	91 (1)	99 (1)	+ 8
Rabbit-nembutal	5	156 (1)	149 (1)	− 7

Mean difference   − 0·44 ± 4·2 (9)
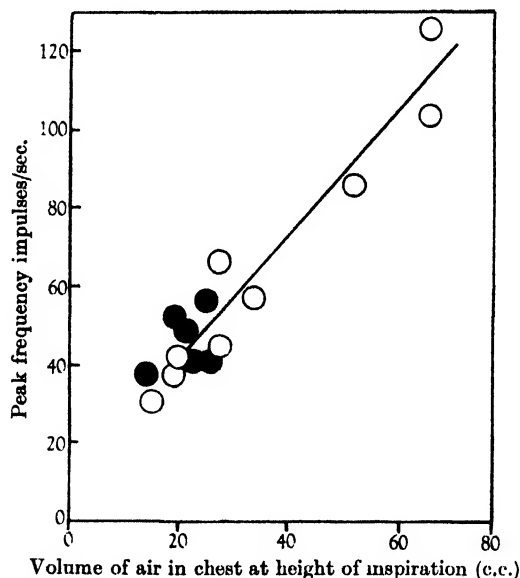
Fig. 1. Relationship between frequency of discharge of stretch receptor and height of inspiration. Spontaneously breathing decerebrate cat. Hollow circles before, black circles after starch injection.

In the second series of experiments the animals breathed spontaneously. Deep breaths were induced by the inhalation of carbon dioxide so that the rate of discharge of a stretch ending could be measured under widely varying degrees of chest expansion (Fig. 1). In conformity with the findings of Adrian (1933), a linear relation was found to exist between the volume of air in the

lungs at the peak of inspiration and the maximum frequency of discharge. In the experiment represented by Fig. 1, the respiratory rate rose from 26 to 52/min., and the functional residual air decreased, following the injection of starch, but the properties of the ending did not change. Likewise in three similar experiments, one of which is represented by Fig. 2, there was no change in the sensitivity of the endings.

The stretch receptors are characteristically silent during expiration, but, in some, a few impulses are discharged at each heart beat. After the injection of starch, the stretch receptors frequently adopt such a rhythm, discharging 1-5 impulses at each heart beat throughout expiration.
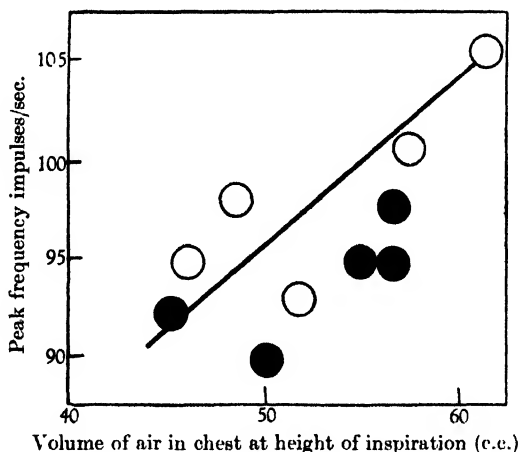
Fig. 2. Relationship between frequency of discharge of stretch receptor and height of inspiration. Another spontaneously breathing decerebrate cat. Hollow circles before, black circles after starch injection.

A few fibres were encountered which discharged discrete volleys of impulses at each heart beat during both inspiration and expiration. Unlike the stretch receptors these endings do not respond to an artificial inflation by discharging a continuous series of action potentials, and their behaviour suggests that they are located on the walls of blood vessels. One bundle of fibres encountered in an artificially ventilated cat probably had a depressor function, for its behaviour suggested that the endings were located on the walls of a *systemic artery*. The discharge was systolic, paralleled the systemic arterial pressure, and was uninfluenced by respiration. After the injection of starch, the blood pressure fell and the activity of the bundle decreased. One fibre encountered in a freely breathing decerebrate cat appeared to correspond to an ending on the wall of a *great systemic vein*. Three volleys of impulses, apparently corresponding to the three waves of the venous pulse, were discharged at each heart beat. The discharge varied with the effective venous pressure, increasing with normal inspiration and artificial deflation, decreasing during normal expiration,

and vanishing with positive pressure inflation. A great increase in the *a* and *v* wave volleys occurred after starch embolism corresponding perhaps to an increase in the venous pressure. The problem is complicated however by the fact that the venous pressure sometimes rose and sometimes fell. Two other venous fibres were encountered in cats and their discharge too was increased by the injection of starch. One fibre encountered in a freely breathing decerebrate cat seemed to respond to the *pulmonary arterial pressure.* A volley of 7 impulses
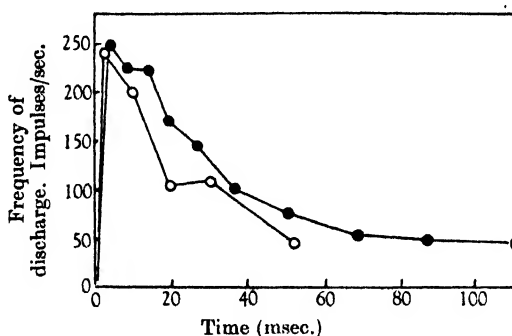
Fig. 3. Discharge of pulmonary arterial pressure receptor. The frequency of discharge declines in any given volley. Hollow circles before, black circles after injection of starch. The frequencies were calculated from the intervals between successive action potentials.

were discharged at each heart beat during inspiration, but only 1–3 impulses per beat during expiration. After the injection of starch the number of impulses in a volley, and the duration of each volley increased (Fig. 3). Such increases were probably related to a rise in pulmonary arterial pressure.

DISCUSSION

The only other direct study of vagal activity following pulmonary embolism of which the author is aware is that of Partridge (1935). In her paper the behaviour of two stretch endings was illustrated. One of these showed no increase in the rate of discharge, whilst the discharge of the other increased from 63 to 100 impulses/sec. As the activity of 13 endings investigated in the present work did not change significantly, it is difficult to believe that the rapid breathing that follows the injection of starch can be due to changes in stretch receptor sensitivity. Further evidence indicating that afferent fibres, other than those responding to pulmonary inflation, were responsible for the rapid breathing was obtained by Torrance (1947). He found that cooling the vagus until the stretch fibres were blocked, did not abolish the reflex respiratory acceleration of starch embolism. It is possible that fibres arising from endings on the walls of blood vessels may be important in this connexion. Three fibres were encountered which appeared to arise from endings on the walls of great systemic veins, a group for which Gernandt & Zotterman (1945) searched

unsuccessfully. The discharge of this group and of a fibre apparently arising from an ending on a branch of the pulmonary artery was increased after the injection of starch. Nevertheless the problem cannot be regarded as solved. Unmyelinated fibres may be important, and the impulses produced would be too small to be detected by present techniques.

SUMMARY

The behaviour of single vagal afferent fibres has been investigated in cats and rabbits, in an attempt to discover the group responsible for the rapid breathing that follows potato starch embolism of pulmonary capillaries. It has been shown that, following embolism:

(1) The peak frequencies of discharge of single stretch endings in artificially ventilated animals show no significant changes.

(2) Stretch ending sensitivity in spontaneously breathing animals is unchanged.

(3) The discharge of some endings, apparently situated on the walls of blood vessels, is increased.

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THE MOTILITY AND VIABILITY OF RABBIT SPERMATOZOA AT DIFFERENT HYDROGEN-ION CONCENTRATIONS

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A considerable literature has accumulated about the viability of mammalian spermatozoa under various conditions of storage in a great variety of media, but no systematic observations seem to have been made on the effects of varying the pH. Cole, Waletzky & Shackelford (1940) added 1–5 % solutions of lactic acid, potassium dihydrogen phosphate or sodium bicarbonate to rabbit semen, keeping it at room temperature for 15 min. to 1 hr. before insemination. They record that no motility was seen outside the pH range 5·0–8·8, and that only slight undulatory non-progressive movements were usually observed outside the range 6·2–7·9. Casida & Murphee (1942) also say that the addition of 5 % sodium bicarbonate solution to rabbit semen often immobilizes the spermatozoa. None of these authors records the actual amounts of the various chemicals that were added to the semen, or seems to have considered the possible influence of changes in tonicity. Baker (1931) must have seen the effects of variations in pH when deciding on his final diluting fluid for mammalian spermatozoa, but does not appear to have recorded his observations. His fluid has a pH of about 8·1, and is said to give optimal conditions for survival.

It is generally agreed that ejaculated spermatozoa are less viable in vitro than those taken directly from the epididymis or vas deferens. In the rabbit, this is confirmed by a comparison of the results of Hammond (1930) and Walton (1930). In Hammond's experiments, semen collected from the vagina after coitus was kept in stoppered tubes for varying periods prior to reinsemination, and in Walton's experiments spermatozoa from the vas deferens were collected under paraffin, and placed in tubes with paraffin seals. Under these fairly comparable conditions (the tests were meant by the authors to be comparable with one another) the fertilizing capacity of Walton's collections was retained considerably longer at low temperatures than was that of Hammond's, ·but the difference disappeared with storage at about 35–37° C. In view of this,

and for convenience in working, it was decided that rabbit ejaculates shielded from too free a gaseous interchange, and kept at room temperature, would suffice for the present studies. From Hammond's figures, it is to be expected that, under such conditions, undiluted semen should retain 50% motility for between 1 and 2 days and lose practically all motility within 4 or 5 days. Hammond found that the fertilizing capacity of semen collections ran parallel with their motility.

MATERIAL AND METHODS

Selection of ejaculates. Semen was collected from rabbits of various breeds with an artificial vagina and the jelly-like fraction, when present, was rejected. Specimens showing an obviously low sperm count or poor motility were also rejected, and thus the results reported below are not obtained from a random sample of ejaculates, but represent the best 50% or so of the several ejaculates usually obtained for test on any one occasion. It was necessary to make these rejections because only a good specimen has sufficient initial motility for the precise detection of the influences which tend to impair the movement of spermatozoa, and provides a sufficient density of cells for making counts in stained preparations at the dilutions used.

Diluents. Very soon after collection (always within 30 min.) the semen was partitioned between various buffered dextrose solutions for observations on viability. These were made in sterile 2 c.c. flat-bottomed glass tubes, each with a second slightly larger tube as a loose cover. As a standard procedure, either 0·05 or 0·1 c.c. of semen, well mixed by gentle pipetting up and down, was placed in 1·5 c.c. of the diluent and again gently mixed by means of a pipette. The resulting suspensions were at a density of from 10 to 60 millions/c.c. The volume of semen used depended on the original volume of the ejaculate, as observations in any one replication of a test were made with a single sample of semen. The diluents all contained 3% of dextrose, 0·2–0·4% of sodium chloride, and sufficient buffer to bring them to a tonicity between that of 0·9 and 1·0% sodium chloride. Preliminary tests of the effect of varying the tonicity of Baker's solution showed that, if anything, a slight increase in the sodium chloride content was beneficial to rabbit spermatozoa. The buffers used were: (*a*) sodium mono- and dihydrogen phosphate mixtures; (*b*) glycine acetate phosphate buffer (Northrop & de Kruif, 1921–2); (*c*) Sørensen glycine (glycocoll) buffer (as given in Clark, 1920); (*d*) Sørensen borate buffer (Clark, 1920); (*e*) sodium carbonate and bicarbonate mixtures. The pH of buffers or semen-buffer mixtures was determined with the B.D.H. capillator outfit, sometimes checked by a reading with the glass electrode.

Motility index. For the determination of motility, a drop of spermatozoal suspension was placed on a glass slide and examined under the microscope within a few minutes.

The usual method of scoring the activity of spermatozoa is to assess the percentage motility. This may be done by eye, or by more elaborate techniques by which the number of immotile spermatozoa is counted at a standard dilution, the specimen killed entirely, and then a full count made. None of these methods seemed adequate for the present purpose, as a specimen may continue to give approximately the same count of immotile cells over a long period, while those remaining motile are in fact becoming progressively less active. Occasionally a semen specimen exhibits a strong contrast between a number of immotile and apparently dead cells and a residue of actively motile cells, even after long storage, but the far commoner picture is of a general damping-down of activity culminating in a high percentage of cells with vibratile tails and stationary heads. In view of this, the following scheme (p. 473) was substituted in order to assess motility. These were found to be grades of activity which are readily carried in the mind and can be applied with little indecision to any specimen with which the observer is confronted, even in the absence of an opportunity for direct comparison. Moreover, it rarely happens that a specimen does not fall into one of the grades listed. When a series of tubes exhibits various grades of activity simultaneously, it is possible, but of doubtful general use, to interpolate quarter-grades to indicate differences between specimens showing very similar activity.

Index of motility	State of spermatozoa
4	Full activity, but there may be up to 30 or 40% of dead cells as in the fresh ejaculate
3½	A detectable damping of activity compared with 4
3	Sluggish, rate of motion about two-thirds that of 4
2½	Most cells progressing but many stationary with tails vibrating
2	Most cells stationary, many with tails vibrating
1½	Many motionless, none or almost none actively progressing
1	Hardly any motility, only tails moving (very rarely an occasional cell in actual motion)
½	Only a few cells per field showing any movement
0	Completely motionless

Staining methods. Lasley, Easley & McKenzie (1942) describe a method of staining semen smears which results in the living cells remaining unstained against a stained background of seminal debris, while the dead cells stain with varying degrees of intensity. These authors noted that several stains could be used, but the best results were obtained with a mixture of water-soluble eosin and opal blue. In the present investigation, it was found that eosin, although giving apparently excellent results, is unsuitable unless the slides are examined immediately, as within a few hours the once-living spermatozoa are stained by diffusion of the dye from the surrounding material. Equally good and more permanent results were obtained with a saturated aqueous solution of Congo red or a 1% aqueous solution of aniline blue.

A droplet of neat or diluted semen is placed on a clean slide and mixed with an approximately equal quantity of stain by means of a glass rod. The mixture is smeared immediately over the surface of the slide and dried rapidly but very gently, high over a flame, care being taken not to heat the slide appreciably. The whole process is complete within a minute. Slides may be examined without further preparation under the oil immersion lens, or may be rendered permanent by the application of a drop of Canada balsam and a cover-slip. If not made permanent, the preparation does not keep for more than a few days, as the smear is an excellent medium for the growth of bacteria and moulds.

All results reported here were obtained with Congo red, which is generally to be preferred to aniline blue because it is easier to see the unstained spermatozoa when this dye is used. Aniline blue gives a better staining of dead cells, but does not colour the background sufficiently well for one to be sure that unstained cells are not missed in making counts. That the stain does in fact differentiate between living and dead spermatozoa was checked by making smears of suspensions killed by heat. Immediately after being heated to 56° C. for a few minutes all cells in fresh suspensions of active spermatozoa are stained, whereas, before heating, the suspensions show a high percentage of living (unstained) cells. The reliability of the method is discussed in detail below.

RESULTS

The stability of buffered suspensions. The mean pH of 24 samples of rabbit semen was 7·6, with a standard deviation of 0·32. The extremes were 7·0 and 8·2. It was to be anticipated that a pH range covering perhaps 5·0–9·0, or even more, would be necessary for the exploration of the effects of hydrogen-ion concentration on viability. With this in mind, early trials were made with the glycine buffers, but both proved unstable at high pH levels. The systems eventually employed as a routine were (*a*) phosphate buffers for the pH range 5·0–8·0 approximately, and (*b*) bicarbonate-carbonate buffers for the range

8·9–11·0 approximately. The apparent gap between pH 8·0 and 8·9 was in practice bridged by the buffering action of normal semen, which sometimes shifted the pH of the carbonate system to as low as 8·1 or 8·2. These latter buffers proved more stable than others and did not exhibit any signs of toxicity (as did, for instance, borate systems). The greater relative stability of the carbonate system is shown in Table 1.

TABLE 1. Stability of buffered alkaline suspensions of rabbit spermatozoa

Buffer	Intended pH	Actual pH of suspension on day			No. of samples
		1	2	3	
Glycine-acetate-phosphate	7·5	7·4–7·6	7·3–7·6	6·6–7·1	4
	10·0	8·8–9·6	8·7–8·8	7·4–8·3	4
Carbonate-bicarbonate	8·9	8·2–8·6	8·1–8·4	7·6–7·9	3
	9·6	9·7–9·8	9·0–9·8	8·8–9·5	7

Even with the most stable buffers, some tests had to be rejected because changes in pH were too wide to be acceptable. It was rare for such changes to occur within the first 12 hr., and equally rare for them not to occur within 3 days. Fortunately, much of the later work involved measurements of motility within the first few hours only, and thus pH changes were not a trouble. Most of the work recorded here, however, covers test periods of up to 48 hr., and the rejection of tubes in which the pH did not remain reasonably constant has so upset the balance of tests that it has been impossible to analyse the results in as efficient a way as was hoped. It has not proved possible, for instance, to eliminate differences between ejaculates in estimating the effects of pH on motility or survival rate.

Effect on motility. Assessments of motility were at first made at varying intervals after the start of a test. It was soon found, however, that observations at $\frac{1}{4}$–$\frac{1}{2}$, 1–1$\frac{1}{2}$, 2$\frac{1}{2}$–3, 4–4$\frac{1}{2}$ and 5$\frac{1}{2}$–6 hr. were adequate for differentiating the effects of pH when the range studied was wide. Further observations were made at 24 and 48 hr. when necessary. Table 2 is an example of the scoring of a test.

TABLE 2. Motility indices from one replication of a test of the effects of pH on the activity of rabbit spermatozoa

	Buffer			
	Baker's solution	Glycine-acetate-phosphate		
pH of suspension at start	7·3	5·3	7·4	8·8
Score at: 0·5 hr.	4·0	0·0	4·0	3·5
1·5 ,,	4·0	0·0	4·0	3·5
3·0 ,,	4·0	0·0	4·0	3·5
4·2 ,,	3·5	0·0	3·5	3·0
5·5 ,,	3·5	0·0	3·0	2·5
24·0 ,,	2·5	0·0	2·5	1·0
48·0 ,,	1·5	0·0	2·0	1·0
pH of suspension at 48 hr.	7·2	5·8	7·1	8·3˙

The suitability of different buffering systems was assessed (in addition to considerations of their stability) by adding the total motility scores for the first 6 hr. and comparing that for each buffer with the results from control tubes containing Baker's solution. The results, taken only from tubes in which the pH did not change by more than 0·2 over the 6 hr. are shown in Table 3. Some later results with carbonate buffers have the phosphate system as control, it being virtually equivalent to Baker's solution. Table 3 lists only the preliminary tests with various buffer systems. When the influence of pH as

TABLE 3. Total motility indices with different buffers during the first 6 hr. of a test

Test no.	Baker's solution (pH of suspension 7·3–7·9)	Buffering agent	pH of suspension			
			6·4–6·5	7·1–7·9	8·5–9·0	9·6–9·8
1	11·0	Glycine-acetate-phosphate	7·0	16·5	14·0	—
2	15·5	Glycine-acetate-phosphate	9·5	15·5	15·0	14·5
3	12·5	Glycine-acetate-phosphate	—	12·0	14·0	—
		Glycine (Sørensen)	—	—	12·5	7·5
4	19·0	Glycine-acetate-phosphate	—	18·5	16·0	—
5	11·0	Glycine-acetate-phosphate	—	10·0	8·0	—
6	19·5	Bicarbonate-carbonate	—	—	18·5	17·5
7	—	Phosphate	11·5	20·0	—	—
		Bicarbonate-carbonate	—	—	17·0	15·0
8	—	Phosphate	12·0	17·5	—	—
		Bicarbonate-carbonate	—	—	17·0	13·8
9	—	Phosphate	12·3	18·3	—	—
		Bicarbonate-carbonate	—	—	15·5	13·8

observed within the same buffering system is taken into account, it will be seen that these buffers are about equally suitable for use. The differences between tests (in which different ejaculates were used on different days) are large. At a pH of less than about 5·8, no motility was seen, although revival of the spermatozoa was possible (see below). On the other hand, the spermatozoa can clearly tolerate a considerable degree of alkalinity.

Table 4 gives the mean motility index at different times after the start of the test, together with the standard error of that mean, for various hydrogen-ion concentrations.

TABLE 4. The effect of pH on the motility of rabbit spermatozoa in vitro

pH of suspensions	No. of tubes	Index of motility at hours				
		0·5	2·5	6	24	48
Up to 5·8	8	0·0	0·0	0·0	0·0	0·0
6·4–6·5	3	3·7±0·33	2·5±0·27	1·0±0·0	0·3±0·17	0·0
7·2–7·9	10	4·0±0·0	3·5±0·17	2·6±0·16	1·2±0·44	0·6±0·28
8·5–8·9	5	3·5±0·27	2·8±0·37	1·7±0·46	0·7±0·30	0·2±0·20
9·7–9·8	6	3·5±0·26	2·4±0·48	1·2±0·18	0·0	0·0
10·2 approx.	5	3·5±0·25	1·8±0·12	0·6±0·40	0·0	0·0

With the restriction that only suspensions in which the pH did not change by more than 0·5 in the course of 2 days are included (it was in fact nearly always

within 0·3 of its initial value), the data in Table 4 comprise all results obtained with the glycine, phosphate and bicarbonate-carbonate systems, prior to those to be reported in a further communication dealing with other variables.

The mean motility indices for pH 7·2–10·2 are also shown in Fig. 1 (those for pH 6·4 are omitted for clarity), and have been fitted, by eye, by a series of smooth curves. The standard error of each point is that applicable to the group of ejaculates with which the tests were made—ejaculates with an initial motility index of 4·0—and would be wider if entirely random samples had been made. However, the object of the tests was not the determination of the extent of variation in a normal population, but the effect of pH on active and fully-motile spermatozoa.
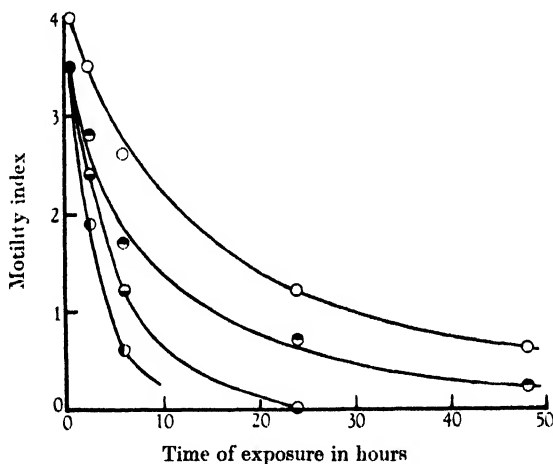
Fig. 1. Mean indices of motility of suspensions of rabbit spermatozoa after exposure to different hydrogen-ion concentrations (alkaline pH's). ○—○ pH 7·5; ◑—◑ pH 8·7; ◕—◕ pH 9·7; ◑—◑ pH 10·2.

Spermatozoa which are completely immotile are not necessarily dead. At pH 4·4–4·6, no motility is seen, but on adding excess of Baker's solution the spermatozoa are seen to revive very considerably unless they have been in the acid buffer for more than about 1 hr. A longer period during which revival is possible is found with progressively higher levels of pH. Above pH 6·5, little or no additional activity is seen when moribund suspensions are diluted with Baker's solution or with other buffered media designed to bring the pH to near 7·5; even above pH 9·8, revival is not particularly evident, but may be seen for up to about 6 hr. at pH 10·0 approximately. Sufficiently high degrees of alkalinity to inactivate the spermatozoa immediately have not been investigated, largely because of difficulties in finding a suitable buffer. In Table 5, the data concerning this phenomenon are summarized.

TABLE 5. The revival of rabbit spermatozoa after suspension in vitro
at various hydrogen-ion concentrations

| pH of suspension | Time of exposure (hr.) | Motility indices | | No. of samples examined |
		Initial	After added Baker's solution	
4·4–4·6	Up to 0·75	0	2–3	2
	1 25	– –	0	—
5·3–5·4	Up to 1·0	0	2·5–3	2
	1 5	---	1–1·5	—
	3·0	—	0	—
5·5–5·8*	Up to 1·25	0	2·5–3	2
	3·0	– ·	1	—
	4·0	—	0·5	—
	5·0	—	0	—
6 4–6·5	4–6	1	1·5 -2 5	5
	24	0	0·0- 1·0	---
Above 7·0	At any time	—	Increases of 0·0- 0·5, with Baker's solution or other buffers	—

* The specimen immotile at pH 5·8 would appear to be exceptional, as in subsequent work other ejaculates have retained some degree of motility for a few hours at this pH.

Effect on mortality. Smears from each tube under test were taken at approximately 0·5, 2·5, 6, 24 and 48 hr., or at 1·5 and 3·5 instead of 2·5 hr., in the case of the more acid suspensions. In addition, one or more smears were made from each fresh sample of semen before dilution. One hundred cells were counted on each slide, and the count was sometimes duplicated from the same slide or from a different slide made at the same time, as a check on technique. The variance of such counts can be partitioned in theory into two parts; the first is that associated with differences between samples, the second is that associated with random sampling. The second should, if the technique is entirely satisfactory, be determinable from the variance of the binomial distribution.

In the present series, technique was not entirely satisfactory. The mean variance for duplicate counts from the same slide was approximately 2·8 times that expected from the binomial distribution, while the mean variance for counts on different slides made at the same time was approximately 3·1 times that expected from theory. This indicates that the fault lies in the preparation or examination of the slides, different regions of one slide tending to be as variable as regions taken from entirely different slides. No significant improvement occurred with practice or after slight modifications in technique, and as the standard errors concerned were, although larger than expected, small enough to be unimportant in comparison with differences between ejaculates, no further steps were taken. Thus, the expected variance of counts of 100 cells at a mean of 72·5% alive is 19·9, the observed variance from duplicate counts was 62, while the variance between the ten tubes which gave this mean was 1165. These were ten suspensions of different ejaculates at the same pH, 24 hr. after the start of the test. Similar figures were obtained with all tests.

The estimated percentage of living (unstained) spermatozoa in the original ejaculate varied between 59 and 90 %. The percentages estimated from samples taken from dilutions at various subsequent times were corrected so that the initial percentage living was equated to 100. The results are shown in Table 6, which lists the same series of tests as in Table 4. The standard error of each mean percentage of living cells follows that mean.

TABLE 6. The effect of pH on the mortality of rabbit spermatozoa in vitro.
All percentages are adjusted to an initial percentage of 100 in the fresh ejaculates

pH of suspensions	No. of tubes	Percentage of living spermatozoa at hours						
		0·5	1·5	2·5	3·5	6	24	48
4·4–4·6	4	41±3	11±2	—	6±2	1±0·6	0	0
5·2–5·8	4	97±3	91±5	—	14±3	5±2	1±0·5	0
6·4–6·5	3	77±12	—	74±3	—	40±18	11±0·3	—
7·2–7·9	10	95±4	—	95±6	—	79±8	73±11	26±9
8·5–8·9	5	78±9	—	74±7	—	81±12	49±17	7±6
9·7–9·8	6	84±10	—	72±6	—	54±3	17±10	0·3±0·9
10·2 approx.	5	75±9	—	65±8	—	49±17	8±5	0

It will be noted that these means tend to be particularly variable at and after 6 hr. for pH levels above 6·4—a reflexion of the differences between ejaculates, which become more apparent later in the tests, and which cannot be systematically examined in the analysis owing to factors already discussed. However, as Figs. 2 and 3 demonstrate, the general picture is quite clear. In acid suspensions, the spermatozoa die rapidly. In alkaline suspensions they survive—just as they remained motile—considerably longer. Half the spermatozoa originally alive are killed at approximately the following times at various pH levels:

pH	4·5	5·5	6·5	7·5	8·7	9·7	10·2 approx.
50% mortality (hr.)	0·5	2·5	4·5	32	21	7·5	5·5

Spermatozoa in the original counts summarized in Table 6 were classed under four headings: (a) unstained, (b) head only stained, (c) tail only stained, and (d) both head and tail stained. Only those in class (a) have been scored as alive. Few cells show head staining alone, but it was observed that, as the cells die, their tails usually stain first, followed by the heads. This occurs independently of pH; a typical series from one test is shown in Table 7.

TABLE 7. An illustration of tail staining in spermatozoa as death occurs

Time after start of test (hr.)	pH 4·4				pH 7·8				pH 9·0			
	U	H	T	B	U	H	T	B	U	H	T	B
1½	12	4	30	54	—	—	—	—	—	—	—	—
3½	3	3	16	78	64	10	7	19	77	12	9	2
6	1	3	12	84	47	14	23	16	38	8	16	38
24	0	2	0	98	40	5	31	24	9	1	50	40

U = unstained; H = head stained; T = tail stained; B = both stained.

Fig. 2. Mean percentages of living rabbit spermatozoa after exposure to different hydrogen-ion concentrations (acid pH's). (Initial % living = 100.) O—O pH 7·5; ●—● pH 6·5; ◐—◐ pH 5·5; ◖—◖ pH 4·5.
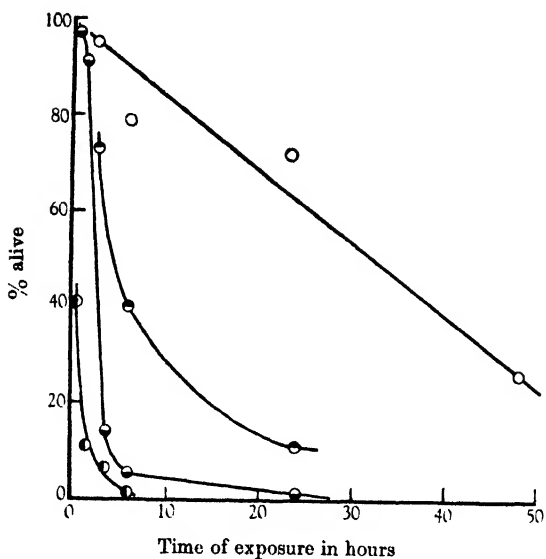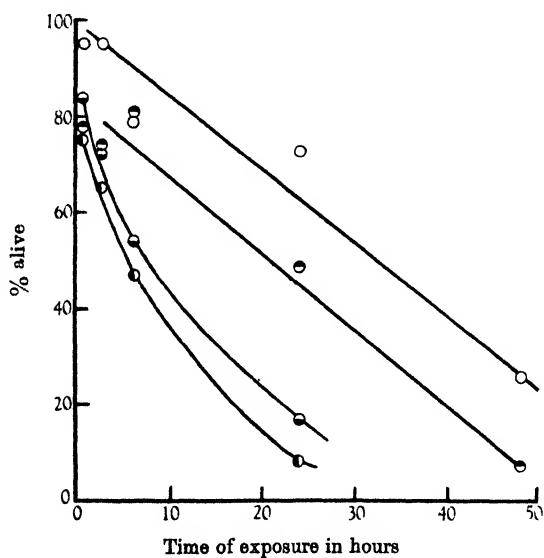

Fig. 3. Mean percentages of living rabbit spermatozoa after exposure to different hydrogen-ion concentrations (alkaline pH's). (Initial % living = 100.) O—O pH 7·5; ●—● pH 8·7; ◐—◐ pH 9·7; ◖—◖ pH 10·2 approximately.

No increase in head staining occurs in this example and the trend of events is clearly death of the tail followed by death of the head. This is to be expected, as the cytoplasm of the tail is both furthest removed from the nucleus and most effectively exposed to the surrounding medium.

Relationship between motility and viability. The absence of movement does not mean that a spermatozoon is dead. Thus, spermatozoa at pH 5·5 are immotile, but may be revived if they have not been too long in acid. On the other hand, the data indicate that this immotility is soon followed by death. At pH 4·4–4·6, the cells cannot be revived by the addition of excess of Baker's solution after about 1 hr., at which time 80% stain with Congo red. At pH 5·2–5·8, they cannot be revived after about 3–4 hr., at which time they are again nearly all dead by the staining criterion. In more neutral or alkaline suspensions, revival on the addition of Baker's solution or other more acid buffered dextrose solutions is less striking or even absent, and under these conditions motility continues more or less parallel with the percentage remaining alive. A motility index of unity would appear to correspond roughly with a 50% mortality, the actual estimated mortalities at this level being:

pH	6·5	7·5	8·7	9·7	10·2 approx.
% dead at unit motility index	60	45	41	49	47

This approximate relationship is independent of pH between the limits shown.

It will be noted, however, that a motility index of unity implies no, or practically no, progressive motion on the part of the spermatozoa—those not yet dead are in process of dying. Suspensions still showing active movement, with an index of 2·5 have, however, suffered fair mortality in addition to that already observed in the fresh semen:

pH	6·5	7·5	8·7	9·7	10·2 approx.
% dead at a motility index of 2·5	26	13	21	28	32

In scoring motility we are, therefore, employing a sensitive index covering the mortality range up to about 50% in suspensions above pH 6. Beyond this, the motility index is relatively insensitive, and in suspensions below about pH 6 it is useless.

<div style="text-align:center">SUMMARY</div>

1. The motility and survival rate of rabbit spermatozoa suspended at room temperature in buffered dextrose solutions has been studied at various levels of pH between 4·4 and 10·2. Suspensions were made only from active ejaculates with high counts, and were at approximate concentrations of between 10 and 60 million cells per c.c. Various buffers were used, but the proportion of dextrose was kept constant at 3% and the tonicity at between that of 0·9 and 1·0% sodium chloride.

2. Motility was scored by means of an arbitrary scale in which fully active specimens were given a rating of 4. The percentage of living spermatozoa was

estimated from smears stained with Congo red, in which cells alive at the time of making the smear remain unstained. Standard errors of estimate are given in the text.

3. Rabbit semen has a pH of between 7·0 and 8·2; the mean of 24 samples was 7·6. The spermatozoa are much more sensitive to acidity than to alkalinity. Below a pH of about 5·8 they are immotile and die rapidly, whereas at a pH of 9·5–10·0 they remain motile and survive for several hours, although with depressed motility. A motility index of unity corresponds roughly with a 50% mortality at pH levels between 6·5 and 10·0. The mean times at which unit index was reached and at which 50% of cells originally alive had died were:

pH	Index of unity (hr.)	50% mortality (hr.)
4·4–4·6	Immotile	0·5
5·2–5·8	Immotile	2·5
6·4–6·5	6	4·5
7·2–7·9	29	32
8·5–8 9	15	21
9·7–9·8	7	7·5
10·2 approx.	4·5	5·5

It should be noted, however, that unit motility index implies that hardly any activity is seen in the suspension, most cells being motionless, the rest moving their tails feebly.

4. Spermatozoa rendered immotile by acidity may be revived by the addition of excess of Baker's solution (pH 8·1 approximately) if they have not been too long at an adverse pH. The times at which they may be so revived correlate well with the mortality data, and no revival was seen where the death rate, as estimated from smears, exceeded 80%. Little or no revival is seen on adding Baker's solution or other more acid media to alkaline suspensions, in which the spermatozoa remain motile (if feebly) until dead or moribund.

5. As death occurs, at all pH levels, the tails of the spermatozoa tend to stain before the heads.

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THE SMOOTH MUSCLE CONTRACTING EFFECTS OF VARIOUS SUBSTANCES SUPPOSED TO ACT ON NERVOUS STRUCTURES IN THE INTESTINAL WALL

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Recently, Ambache (1946) has analysed the contractions of the isolated mammalian intestine produced by acetylcholine, histamine, potassium and barium ions. On the basis of his results, he concluded that the contractions produced by small doses of acetylcholine, by potassium ions and by barium ions were not due to a direct effect of these substances on the muscle fibres but to an action on the nervous elements present in the preparations. Small doses of acetylcholine were said to act like nicotine by stimulation of the nerve cells of the plexus of Auerbach, whereas barium and potassium ions were said to act by release of acetylcholine from the nerve fibres of Auerbach's plexus. Histamine was stated to have an action like potassium and barium in addition to a direct smooth muscle stimulating effect. The importance of such a new conception of the pharmacological effects of these substances on the isolated intestinal preparation made it desirable to repeat the experiments. When this was done no conclusive evidence was obtained in favour of the theory that these substances owe their muscle contracting property to an action on nervous structures in the intestinal wall.

The conclusion that small doses of acetylcholine have a nicotine-like action on the intestine was based by Ambache on the observation that the sensitivity of the gut to acetylcholine diminishes greatly after paralysing doses of nicotine. No controls, however, were made to ascertain if this change in sensitivity was specific for acetylcholine. The conclusion that the effects of histamine, potassium and barium ions was 'indirect' was based primarily on experiments with cooled preparations. According to Ambache, cooling the intestinal preparation reduces its sensitivity to histamine and abolishes the response to potassium and barium, whereas it does not affect the sensitivity to substances which stimulate smooth muscle directly. However, his experiments do not provide evidence for the second conclusion since it was not shown that the response to substances stimulating muscle directly remains unaffected by

cooling. The only substance used for this purpose was acetylcholine. A diminished response was obtained with this substance after cooling the intestine, but was attributed to a loss of the assumed nicotine action and not to impairment of the muscle fibre itself.

The hypothesis that acetylcholine is released by histamine, by potassium ions and by barium ions was supported by two additional observations: (1) histamine and barium ions were found to accelerate the synthesis of acetylcholine; and (2) histamine, potassium ions and barium ions were found to act more strongly when given with eserine. On repetition of these experiments, no evidence could be obtained that histamine and barium ions accelerate the synthesis of acetylcholine and the results obtained with eserine, when carried out with necessary controls, were found to supply no evidence in support of Ambache's hypothesis.

In the present experiments the hypothesis of a release of acetylcholine by histamine, potassium and barium was also tested by studying the response to these substances by the muscle when paralysed either by atropine or by benadryl (dimethylaminoethylbenzhydryl ether hydrochloride).

METHODS

The experiments were carried out on the isolated preparation of the guinea-pig's ileum and the rabbit's jejunum suspended in 10 c.c. Mg-free Tyrode's solution. The contractions of the fibres of the longitudinal muscle layer were recorded by a Lovatt Evans frontal writing lever. The bath was emptied by overflow and the substances were added in 0·2–0·4 c.c. volume with a syringe; air was bubbled continuously through the Tyrode solution, the temperature of which was between 30 and 35° C.

The cooled preparations were kept in Mg-free Tyrode's solution at 0–2° C. for varying times. Before use, any secretion accumulated in the lumen was pressed out gently.

Acetylcholine and choline were used as chlorides, histamine as dichloride and pilocarpine as nitrate. The values refer always to the salt. The values for barium chloride refer to $BaCl_2$, $2H_2O$. A sample of ethylal-propanediol-trimethyl ammonium iodide (2268 F) was kindly supplied to us by Sir Henry Dale. This substance acts like muscarine and has no nicotine-like effects on ganglion cells (Fourneau, Bovet, Bovet & Montezin, 1944).

Synthesis of acetylcholine. The method used was essentially the same as that described by Ambache. The small intestine of guinea-pigs was removed, washed with saline solution and slit open longitudinally. It was cut into pieces about 1 cm. long and these were distributed among all the samples. For instance, when six samples were set up a piece of intestine about 6 cm. long was cut into six strips and one placed in each flask; then another piece of the intestine was cut up and the strips distributed similarly amongst the flasks. In this way the whole small intestine was used up and the same procedure was adopted with the intestine of a second and third guinea-pig. The strips of each sample were dried between filter-paper and the total tissue of each sample weighed. The amounts of tissue used for each sample varied between 3 and 3·5 g. They were suspended in 5 or 10 c.c. of saline solution with the following composition: 9·2 g. NaCl, 0·42 g. KCl, 0·24 g. $CaCl_2$, 1 g. dextrose in 1 l. H_2O. Eserine sulphate was added to give a concentration of 1 in 200,000 in the samples. In one experiment (No. 5, Table 2) the solution was buffered with phosphate. The incubation was carried out at 37° C. for 40 min. in either air or oxygen. During this time, the samples were shaken continuously. For the extraction 4 c.c. N/3-HCl were added to each sample which was then ground with sand and boiled for 1 min. The samples were cooled and centrifuged;

the residue was washed twice, and the supernatant with the washings made up so that 10 c.c. corresponded to 1 g. of tissue. The samples could be kept in this condition overnight in the refrigerator. The acetylcholine content of the samples was assayed, after neutralization, on the eserinized frog's rectus muscle against solutions of acetylcholine to which had been added equivalent amounts of the same extract after it had been boiled for a moment in alkaline solution and neutralized. This procedure is necessary to obtain reliable quantitative results (Feldberg, 1945).

RESULTS

The response of the isolated intestine to drugs during nicotine paralysis

When a large dose of nicotine is given, a strong contraction ensues, followed after a few minutes by relaxation, despite the fact that the nicotine remains in the bath. When it is washed out and replaced at once by a fresh dose of
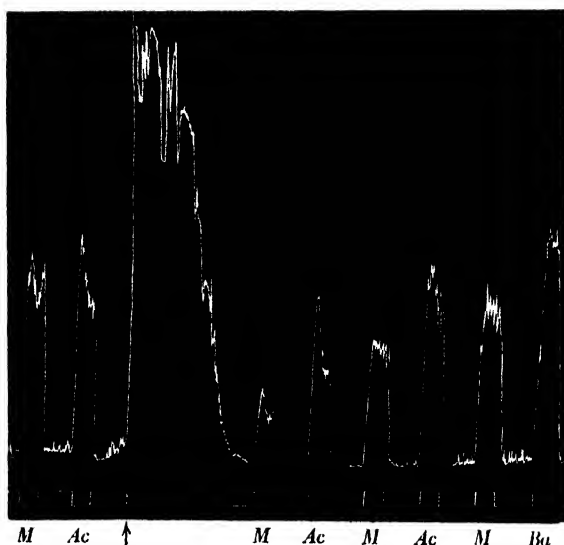
Fig. 1. Contractions of the isolated guinea-pig's intestine in 10 c.c. bath. At *M* $0.025 \mu g$. 2268 F, at *Ac* $0.038 \mu g$. acetylcholine, at *Ba* 1 mg. $BaCl_2$. All substances kept in the bath for 1 min. From the arrow (↑) till the end of experiment the bath fluid contained nicotine tartrate 1 in 50,000.

nicotine, no further contraction occurs, suggesting that the ganglion cells are now paralysed. During the initial phase of the paralysis the response to acetylcholine is sometimes, but by no means always, reduced; but, if so, the responses to other drugs like histamine, pilocarpine or 2268 F are reduced to the same extent. The period of reduced sensitivity is of short duration; within 10-20 min. the preparation gradually regains its original sensitivity to acetylcholine, histamine, pilocarpine and 2268 F, although the nicotine is kept in the bath and the ganglion cells remain paralysed. These results were obtained on the isolated intestine preparation of the rabbit and of the guinea-pig. Fig. 1 is from an experiment on the guinea-pig's intestine and illustrates (*a*) an initial phase of reduced sensitivity after paralysis with nicotine, but for 2268 F as well as for acetycholine; and (*b*) the gradual recovery of the

original sensitivity to both substances, although the ganglion cells remain paralysed. Hence there is no evidence that small doses of acetylcholine owe their stimulating effect on the intestine to an action on ganglion cells. The experiment shown in Fig. 1 also illustrates that nicotine paralysis does not abolish the stimulating action of $BaCl_2$ on the intestine.

Changes in the sensitivity to drugs after cooling the intestine

We have not been able to observe that cooling the rabbit's intestine affects the response to various drugs differently but have found that differences may be detected, although by no means regularly, on the cooled guinea-pig's intestine.

Rabbit's intestine

The cooled preparation, when suspended in the bath, started to contract gradually after an initial period of relaxation. During the initial relaxation, which lasted between 30 and 60 min., the muscle was often insensitive to any drug examined, at least when the cooling had been carried out for several days.

When the contraction started to develop, the muscle became sensitive to the various substances, but there was no clear or regular difference in the degree of the response of the muscle to the various substances. Sometimes it appeared as if the sensitivity of the muscle had been reduced to a greater extent for one substance, sometimes for another. A typical experiment showing such irregularities is given in Fig. 2. Tracing *a* shows the contractions produced on the fresh preparation by $0.1\mu g$. of acetylcholine, 1 mg. of $BaCl_2$, $300\mu g$. of histamine, $2.5\mu g$. of pilocarpine and 10 mg. of KCl. The tracing *b* is taken from an adjacent piece of intestine which had been cooled for 4 hr. From the responses obtained during the initial period of relaxation it would appear that 4 hr. of cooling had affected the sensitivity to acetylcholine and to pilocarpine more than that to $BaCl_2$. When KCl was tested, the muscle had started with its gradual contraction; KCl had practically no effect. It might, therefore, appear as if there were a fundamental difference between the actions of $BaCl_2$ and of KCl, but a different impression was gained when these drugs were retested a little later. Between the two parts of *b* there was an interval of 15 min. during which the muscle contracted to a new level. Now all five substances produced small contractions. Tracing *c* is taken from another piece of the same intestine after 5 days of cooling and after $2\frac{1}{2}$ hr. suspension in the bath. During the initial period of relaxation none of the five substances had caused contraction. Later, when the muscle had partly contracted, it responded again to the different substances but the effect was small as compared with the original effect of these substances. There was practically no difference in the response to $0.1\mu g$. of acetylcholine, 1 mg. $BaCl_2$, 10 mg. KCl and $400\mu g$. of histamine; the sensitivity of the muscle to these substances had been reduced to practically the same extent. Pilocarpine alone was wholly inactive in the original dose ($2.5\mu g$.); it caused a small contraction only when four times the

Fig. 2. Effect of cooling rabbit's intestine, suspended in 10 c.c. bath, on sensitivity to acetylcholine (*Ac*), BaCl₂ (*Ba*), histamine (*Hi*), pilocarpine (*Pi*) and KCl (*K*). (*a*) fresh preparation; (*b*) after 4 hr. cooling; (*c*) after 5 days' cooling. The distance between the base line and the record from the intestine gives the degree of contraction of the muscle. (For details see text.)
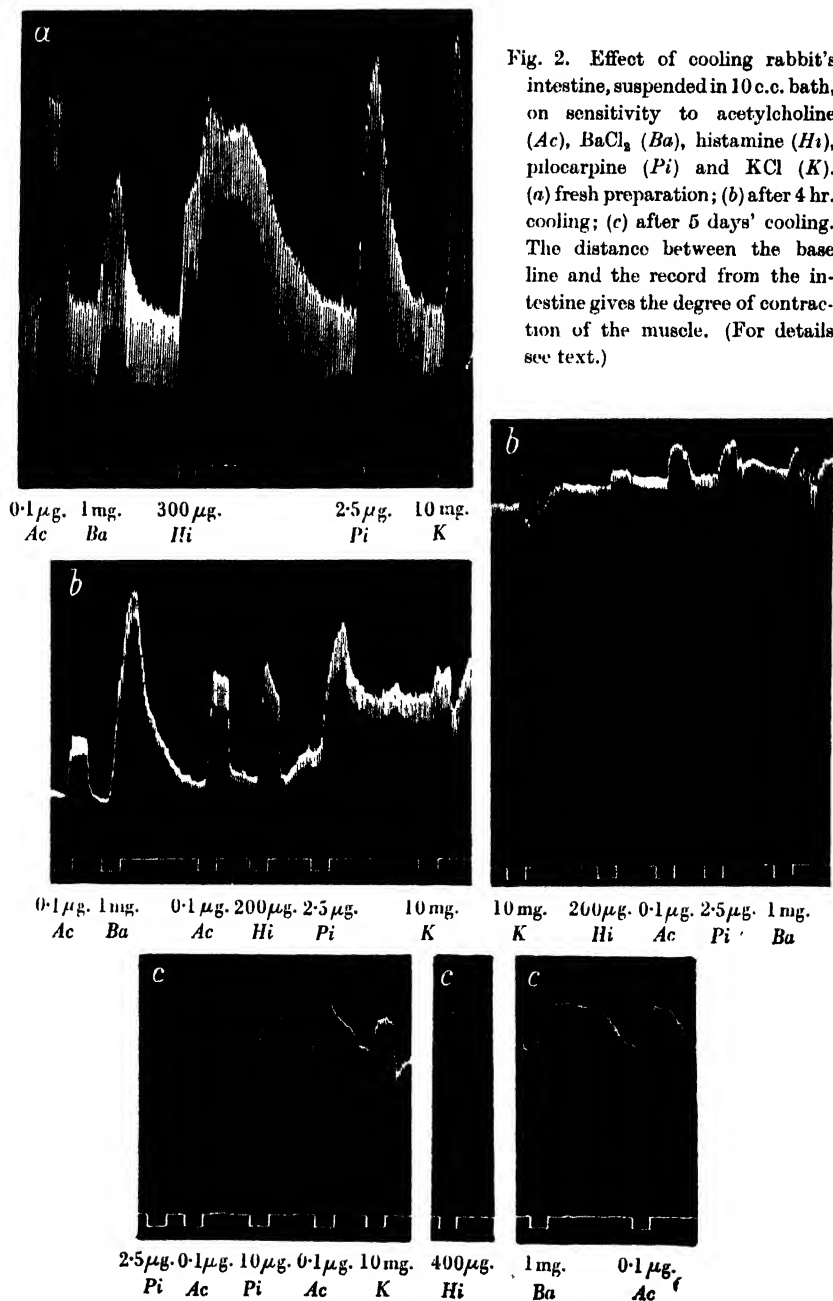
amount was given. This result was not observed in all experiments. After 7 days of cooling the intestine had lost its ability to contract to any of the five substances even when they were given in larger doses and after a long period of suspension in the warm bath.

Guinea-pig's intestine

Unlike the rabbit's intestine preparation, that of the guinea-pig only rarely showed a spontaneous gradual increase in tone when examined after cooling. There was, however, an initial phase, lasting 20–60 min., in which the suspended preparation was more or less insensitive to drugs. Therefore, tests had to be carried out after this initial phase had passed. A quantitative comparison of the effect of cooling on the sensitivity of the muscle to various drugs was found to be extremely difficult and sometimes even impossible on account of the following facts:

(a) The sensitivity to a drug like acetylcholine varies even on the same fresh preparation; first it increases and later it decreases, but these changes are by no means regularly obtained and vary in degree and time sequence from one experiment to another.

(b) The sensitivity of the fresh muscle varies differently in the course of the experiment with different drugs so that a certain dose of one drug may have at one time a stronger, at another a weaker action than a specified dose of another drug.

(c) When the amount of a drug tested is doubled, the increase in the response varies with different drugs. This is shown for KCl and acetylcholine at the beginning of the tracing in Fig. 9 a.

(d) Although it is possible to obtain comparable contractions with repeated administration of the same dose of acetylcholine and histamine, apart from the gradual changes in sensitivity of the muscle, other substances like barium and potassium may give greatly different responses when given repeatedly. A particularly striking instance is shown in the case of barium in Fig. 7 a. BaCl$_2$ was given fourteen times in 2 mg. doses. only every third response being reproduced in the figure. In Fig. 6 are shown several different responses to 10 mg. of KCl, and in the experiment of Fig. 9, 5 mg. of KCl had no effect at the beginning of the experiment, but produced strong contractions of the muscle at the end. Such great differences in the response to KCl may occur even when it is given in succession at 4–5 min. interval.

Despite these difficulties, the response to barium and potassium was found in some experiments to be affected to a greater extent by cooling than that to acetylcholine, histamine and pilocarpine. The experiment of Fig. 3 is given as an illustration, but it must be emphasized that the responses of the cooled muscle varied greatly from experiment to experiment. In detail the results for the different substances were as follows.

Fig. 3. Effect of cooling the guinea-pig's intestine, in 10 c.c. bath, on sensitivity to histamine (*Hi*), acetylcholine (*Ac*), BaCl₂ (*Ba*), KCl (*K*) and pilocarpine (*Pi*). (*a*) fresh preparation; (*b*) adjacent piece after 4 hr. cooling and suspension for 35 min.; (*c*) same piece as in (*b*) after 3 days' cooling and suspension for 20 min.; between the first and second part of (*c*) an interval of 70 min.; (*d*) third piece of same intestine after 5 days' cooling and suspension for 100 min.; (*e*) same piece as in (*a*) after 6 days' cooling and suspension for 60 min. (For details see text.)

Acetylcholine. Table 1 illustrates the fact that cooling for 24–48 hr. may depress the sensitivity of the muscle to acetylcholine. It gives the results from two experiments. A number of contractions were obtained with $0 \cdot 1 \mu$g. of acetylcholine, first on the fresh, and later on the cooled preparation. The

TABLE 1. Effect of cooling the guinea-pig's intestine on the sensitivity of the muscle to acetylcholine and KCl

	Percentage of original response to $0 \cdot 1 \mu$g. acetylcholine (Ac) and to 10 mg. KCl			
Days of cooling	Exp. 1		Exp. 2	
	(Ac)	(KCl)	(Ac)	(KCl)
0	100	100	100	100
1	68	34	—	—
2	8	12	70	24
4	—	—	4	4

average height of the recorded responses was measured and expressed as the percentage of the response obtained on the fresh preparation. The contractions on the cooled preparation were obtained after it had been suspended in the warm bath for at least 30 min. It will be seen that even 24 hr. of cooling may depress the sensitivity of the muscle to acetylcholine. When the dose of acetylcholine was increased it was possible to obtain strong contractions even from the depressed cooled preparation. These results agree with those reported by Ambache, since he used much greater doses of acetylcholine (increases up to 100 times) on cooled than on the fresh preparations. In the experiment of Fig. 3, this depression of the acetylcholine response was only slight even after 3 days of cooling; it became pronounced after 5–6 days' cooling, and after 7 days' cooling the muscle had become insensitive to acetylcholine.

Histamine. As long as the muscle was sensitive to acetylcholine it also responded to small doses of histamine. Sometimes the cooled preparation responded less readily to histamine, sometimes to acetylcholine, and on the same preparation, first the response to one, and later that to the other, might appear to be the more depressed. In general, however, the muscle, on prolonged cooling, became simultaneously less sensitive and finally insensitive to both drugs. This is seen in Fig. 3. The responses to both drugs are reduced only slightly after 3, but greatly after 5–6 days' cooling. After 4 hr. cooling, the response to the first dose of histamine (Fig. 3b) is much more reduced than that to acetylcholine given a few minutes later. This result could easily be taken as evidence for a more pronounced effect of cooling on the response to histamine than to acetylcholine. It is explained, however, by the fact that the muscle was still recovering from its initial phase of insensitivity to drugs, when the record was started. The difference, in fact, disappeared when the two drugs were retested later on in the experiment.

Pilocarpine usually resembled histamine and acetylcholine in the way its action was affected by cooling the muscle. In some experiments, however, the response to pilocarpine was reduced to a much greater extent, and this result was not dependent on the time for which the muscle had been cooled. For instance, in the experiment of Fig. 3 the response to 0.2μg. of acetylcholine and to 10μg. of pilocarpine were comparable on the fresh preparation and after 4 hr. cooling; after 3 days' cooling the effect of 10μg. of pilocarpine was slightly less than that of 0.2μg. of acetylcholine. After 5 days' cooling, 100μg., and after 6 days' cooling only 30μg., were needed to produce the same small effect as 0.2μg. of acetylcholine.

Potassium. The results were not uniform. In some preparations potassium behaved like acetylcholine. The response of the muscle to both substances became gradually smaller on prolonged cooling. This is illustrated in Table 1. It is true that the sensitivity to KCl decreased to a greater extent than that to acetylcholine. Since the concentration gradient of the two substances when given in increasing doses on the fresh preparation is different, such a result is not surprising. The experiment of Fig. 3, on the other hand, illustrates a result in some ways resembling those obtained by Ambache. Cooling for 4 hr. seemed to have depressed the response to 5 mg. of potassium to a much greater extent than that to 0.2μg. of acetylcholine. When the dose of potassium was increased to 7.5 mg. a greater response was obtained than with 0.2μg. acetylcholine. In addition, it was found, in this as well as in many other experiments, that the sensitivity to potassium could be partly restored when it was given in successive doses (see end of tracing *b*). After 3 days' cooling 15 mg., and after 5–6 days' cooling 20 mg. KCl were required to match approximately the response to 0.2μg. of acetylcholine. The response to acetylcholine, however, is also greatly reduced and it must be emphasized that the reduction in the response to KCl was always associated with a general reduction in the sensitivity of the muscle to all drugs examined.

Barium. There is no doubt that cooling affects the response of the muscle to $BaCl_2$ more than that to any other substance so far examined. This is evident from the experiment of Fig. 3. On the cooled preparation a ten-fold increase or more in the dose of $BaCl_2$ only produced a small contraction, but again it will be seen that the effect of cooling on the response to $BaCl_2$ proceeds gradually. In addition it must be stated that, even on the fresh preparation, the effects of very small doses of barium cannot be obtained with regularity when it is given in successive doses.

Eserine

According to Ambache, eserine potentiates the effect, on the isolated fresh intestine, of histamine, KCl and $BaCl_2$. These results were taken as further proof for the view that the three substances release acetylcholine. His experimental procedure, which was imitated closely in the present experiments, was

as follows. Eserine, in a concentration of about 1 in 3·5 millions, was added to the bath and followed usually, after 10–15 sec., by either histamine, BaCl$_2$ or KCl; eserine and the substance to be tested were then kept in the bath for as long as 2 min.

We can confirm Ambache's observation that under these conditions the substances he has examined act more powerfully, but this 'potentiation' by eserine occurred with all muscle-stimulating substances and is no evidence for the theory that they cause a release of acetylcholine.

Ch   Ch   Es+Ch   Ch   Ch   Ch   Ch   Ch   Es
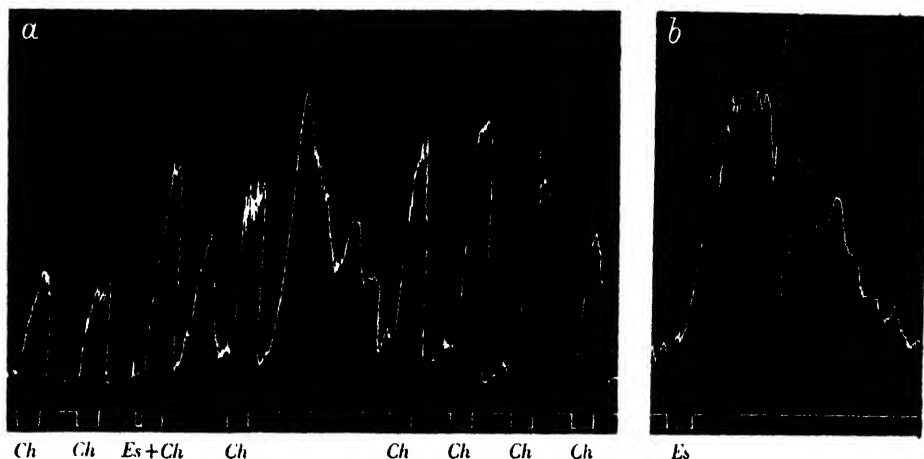
Fig. 4. Contractions of the isolated guinea-pig's intestine in 10 c.c. bath. At (*Ch*) 0·3 mg. choline kept in bath for 1 min.; 15 sec. before the third choline dose 2·5µg. eserine sulphate (*Es*) added to bath and washed out after 75 sec. with the choline. Record (*b*) effect of 2·5µg. eserine added alone and kept in bath for 75 sec. (For details see text.)

When eserine 1 in 4–5 millions, which is even weaker than the concentration used by Ambache, is added to the bath and kept there for 75 sec. there ensues, usually after a latency of 20–40 sec., a gradually developing contraction of the guinea-pig's intestine which may last for several minutes (see Fig. 4 *b* and Fig. 5 *c*). When a muscle-stimulating substance is added to the bath 15 sec. after the eserine, its effect therefore appears to be much greater. This is illustrated for choline in Fig. 4 *a*. If the tracing had been reproduced in such a way as to give the immediate effect of choline only, the tracing would resemble those obtained by Ambache for histamine, barium and potassium. In some experiments the motor activity of the eserine is so pronounced within the first 75 sec., that there is no immediate relaxation when the eserine and the respective muscle-stimulating substance are washed out. This is illustrated for the muscarine-like substance 2268 F in Fig. 5 *a, b*. The so-called 'sensitization' by eserine thus results from the fact that the eserine causes accumulation of acetylcholine, the effect of which is added to that of the respective muscle-

stimulating substance. In Ambache's experiments themselves, there is evidence that the accumulation of acetylcholine by eserine was so great as to produce, by itself, contraction of the intestine. He reproduces three figures (Figs. 8, 11, 16) in which the muscle did not relax completely after the eserine and the respective substance had been washed out.
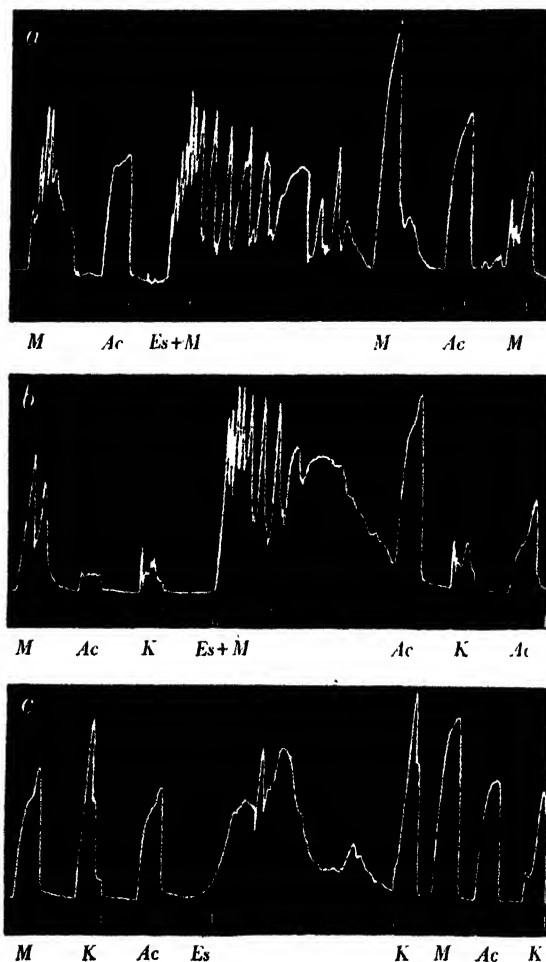
Fig. 5. Contractions of the isolated guinea-pig's intestine in 10 c.c. bath. At *Ac* 0·1 μg. acetylcholine, at *M* 0·1 μg. 2268 F, at *K* 5 mg. KCl kept in bath for 1 min. At *Es* 2 μg. eserine sulphate kept in the bath for 75 sec. but followed, in tracing (a) and (b), after the first 15 sec. by 0·1 μg. 2268 F. (For details see text.)

The effects of various substances have been examined also after the eserine contraction had more or less subsided, and in this condition it was again found that all the muscle-stimulating substances tested produce an increased response which was followed by a period of depression. This is shown in Fig. 5

for 2268 F, acetylcholine and potassium. The 'after sensitization' is almost equally strong with all substances so far tested. Such sensitizations can be explained on lines similar to those suggested for the initial sensitization. The action of eserine has not completely worn off; some accumulation of acetylcholine is still going on, at a rate insufficient to stimulate the muscle. When a muscle-stimulating substance is given during this period, the subthreshold amounts of acetylcholine are sufficient to augment its stimulating effect.

Fig. 6. Contractions of the isolated guinea-pig's intestine in 10 c.c. bath; upper and lower tracing from different intestines. Effect of atropine, 1 in 200 millions on nicotine and potassium contractions. At *Ac* acetylcholine (0·1 µg. in upper and 0·2 µg. in lower tracing); at *Nic* 200 µg. nicotine, at *K* 10 mg. KCl. In upper record the atropine was kept in the bath between the two arrows and in the lower record during (*b*). (For details see text.)

Atropine

Atropine was tested in concentrations just low enough to abolish completely the stimulating effects of small doses of acetylcholine on the intestine. The use of greater concentrations of atropine has been avoided because they render the muscle insensitive to nearly all drugs examined. For instance, Feldberg (1931), found that atropine in concentrations sufficient to abolish an acetylcholine contraction of the guinea-pig's intestine depressed slightly an equally strong

histamine contraction; with higher concentrations of atropine the histamine effect too was abolished (see also Bernheim, 1931).

It might be argued that low concentrations of atropine can abolish the effect of the acetylcholine applied to the outside of the muscle, but not that of the acetylcholine released by the action of drugs from the nervous structures
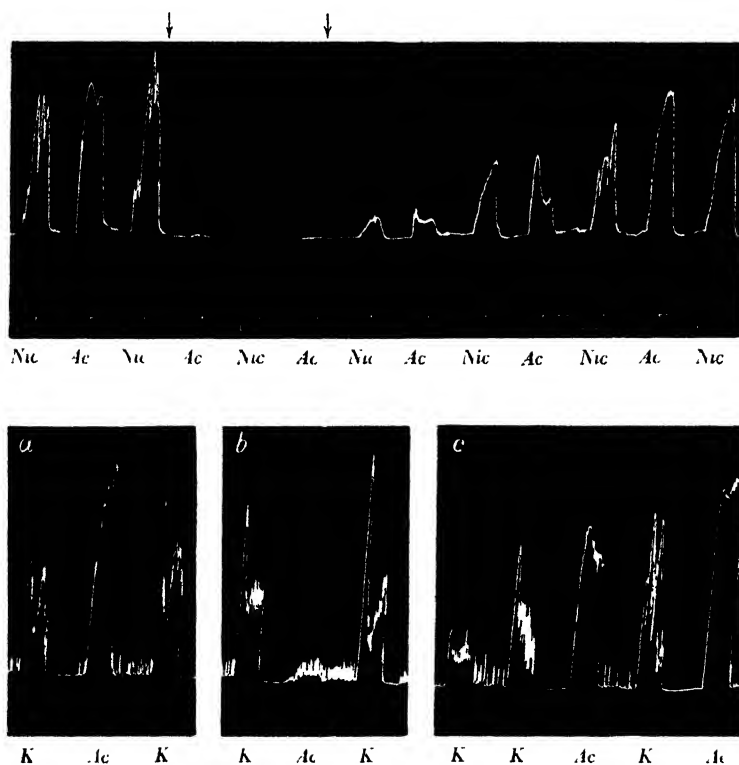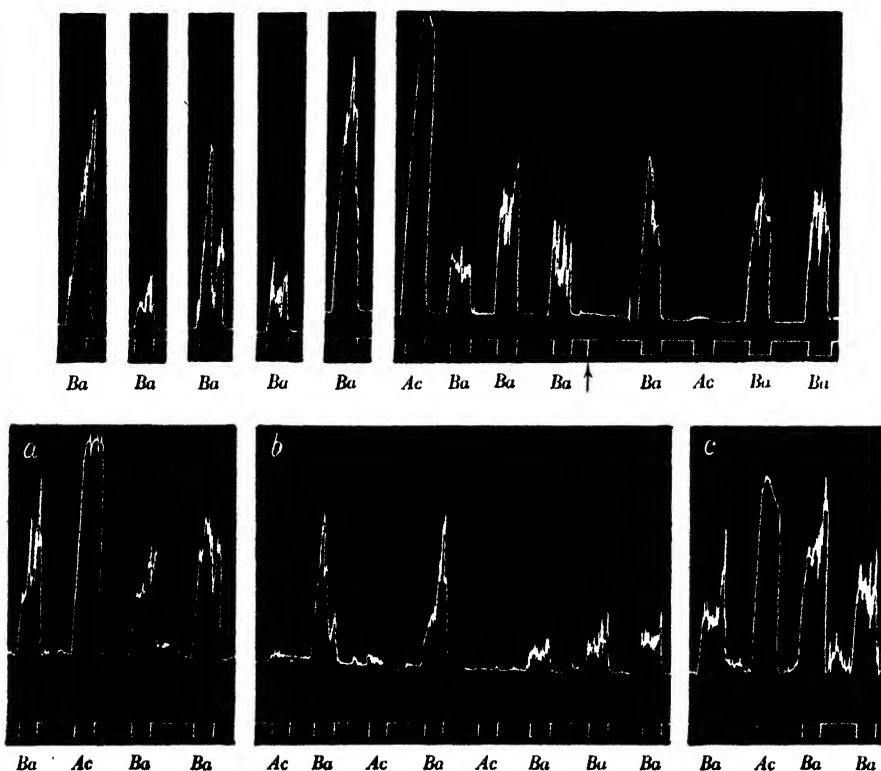

Fig. 7. Contractions of the isolated guinea-pig's intestine in 10 c.c. bath; upper and lower tracing from different intestines. Effect of atropine, 1 in 200 millions, on BaCl₂ contractions. At *Ba*, BaCl₂ (2 mg. upper and 0·8 mg. lower tracing). At *Ac*, 0·2μg. acetylcholine. Atropine given in upper tracing at the arrow (↑) and kept in the bath till end of record and in lower tracing during (*b*). (For details see text.)

present in the intestinal wall. This argument does not hold good. For instance, in the experiment of Fig. 6*a*, atropine, 1 in 200 millions, abolished the equally strong contractions produced by 0·1μg. of acetylcholine and by 20μg. of nicotine; when the atropine was washed out the sensitivity of the muscle to both drugs returned simultaneously. Thus atropine has an identical quantitative effect on the response to acetylcholine, released by the stimulating action of nicotine on the nerve cells in the intestinal wall, and on the response to acetylcholine added to the bath and acting from the outside of the muscle.

Unlike the effect of nicotine, that of potassium is usually resistant to small doses of atropine. In the experiments of Fig. 6 *b* atropine, 1 in 200 millions, had no action on the potassium contraction. In fact, the smallest effect with potassium was obtained several minutes after the atropine had been washed out and the muscle had nearly recovered its original sensitivity to acetylcholine. In some experiments the effect of potassium was not maintained and was reduced after atropine, but, since these changes were not obtained regularly, were not always reversible after washing out the atropine and, in addition, occurred sometimes spontaneously, it is difficult to relate them to the atropine. These results are in agreement with earlier statements on the atropine-resistent action of potassium. They do not support the view that potassium owes its stimulating action on the intestine to the release of acetylcholine.

The great variations in the response of the intestine to repeated administration of barium made it difficult to obtain reliable and regular results. In some experiments, such as the one illustrated in Fig. 7 *a*, the barium contractions were not affected by the atropinization; in others, such as the one illustrated in Fig. 7 *b*, the barium contractions were definitely reduced by the atropine, and, when it was washed out, the muscle regained its sensitivity to barium and to acetylcholine simultaneously. In other experiments, the barium contractions were reduced during atropinization, but not restored when the atropine had been washed out and the muscle had again become sensitive to acetylcholine. There was no parallelism between the effect of atropine on the response to acetylcholine or nicotine, on the one hand, and to barium, on the other. It is not even certain that the irregular depression of the barium response during atropinization must be regarded as an effect of atropine.

Benadryl

Benadryl in low concentrations abolishes the effect of histamine. It is known, and could be confirmed, that such concentrations do not affect the response to acetylcholine. For instance, in the experiment of Fig. 8, $0.15 \mu g$. of histamine gave a stronger contraction of the guinea-pig's ileum than $0.1 \mu g$. of acetylcholine. Benadryl, 1 in 125 millions, practically abolished the response to histamine, but had no effect on the response to acetylcholine. If histamine were to act partly by the release of acetylcholine this result would be difficult to understand. It would then be necessary to assume that the mechanism of the release of acetylcholine by histamine were affected by benadryl. There is no justification for such an assumption, for the benadryl had no effect on the response to $20 \mu g$. of nicotine, the muscle-contracting effect of which equalled that of $0.15 \mu g$. of histamine. Since nicotine acts by stimulating the ganglion cells, and thereby releasing acetylcholine, this result shows that the mechanism of the release of acetylcholine is not affected by benadryl, at least not in the concentration used in this experiment.

According to Loew, McMillan & Kaiser (1946), benadryl also inhibits the action of acetylcholine and of barium on the guinea-pig's intestine, but the concentration required for acetylcholine was 1 in 2·5–4 millions, that for barium about 1 in 100,000. In our own experience a concentration of benadryl which inhibited the response to a small dose of acetylcholine also depressed that to a small dose of barium. For instance, in the experiment of Fig. 9, benadryl, 1 in 1 million, abolished the contraction produced by 0·15μg. of acetylcholine and nearly abolished the equally strong one produced by 1 mg. of barium chloride; 2 mg. of barium chloride, however, produced a much stronger contraction than even 0·6μg. of acetylcholine. It is possible that when large doses of either barium or acetylcholine are used, a much stronger concentration of benadryl is required for antagonizing the effect of barium than that of acetylcholine, but it appears that there is no great difference in the antagonizing action which benadryl exerts on the responses to small doses of acetylcholine and barium respectively on the guinea-pig's intestine.

Fig. 9 also illustrates the fact that benadryl, 1 in 1 million, antagonizes the action of potassium. Instead of the strong contraction produced by 10 mg. of KCl at the beginning of the experiment there was only a relatively small effect, and the contraction was not maintained. Doubling the dose of potassium did not increase the response, but it was interesting that 5 mg. of KCl, which had had only a slight effect when given before the benadryl, was not wholly ineffective when added to the bath containing the benadryl. Such results illustrate

Ac Hi Nic ↑ Hi Ac Nic Hi

Fig. 8. Contractions of isolated guinea-pig's intestine in 10 c.c. bath. Effect of benadryl, 1 in 125 millions (from arrow till end of tracing), on the contractions produced by 0·1 μg. acetylcholine (at *Ac*), 0·15μg. of histamine (at *Hi*) and of 20μg. of nicotine (at *Nic*).

the difficulty of an exact quantitative comparison of the action of benadryl on the responses to different substances. It seems certain, however, that benadryl is a more powerful antagonist of acetylcholine than of potassium. This difference becomes particularly clear after the benadryl had been washed out. During the ensuing period of gradual recovery (Fig. 9c) the sensitivity of the muscle to potassium returned earlier than that to acetylcholine. At a time when the response to acetylcholine was still reduced, the muscle was found to be more sensitive to potassium than it had originally been, and to contract strongly to as little as 5 mg. of KCl. Fig. 9 illustrates further that nicotine, unlike potassium and barium, was affected by benadryl to approximately the same extent as was acetylcholine. The experiments with benadryl, therefore, do not support the view that the contractions produced by potassium and barium result from a release of acetylcholine.

Synthesis of acetylcholine in strips of the guinea-pig's intestine

According to Ambache, histamine and $BaCl_2$ accelerate the synthesis of acetylcholine which occurs in strips of the guinea-pig's small intestine incubated
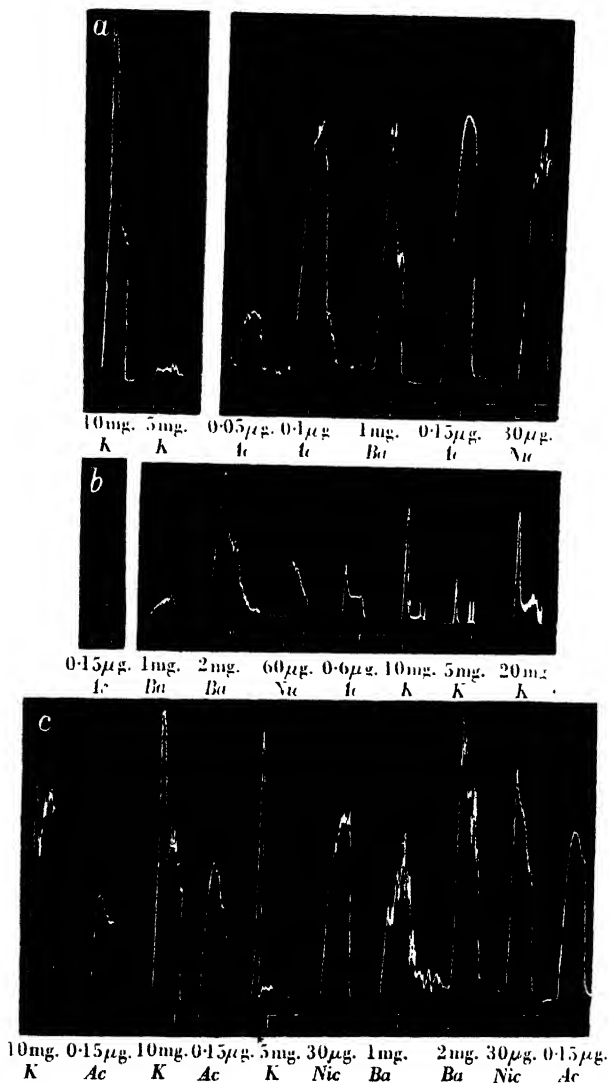
Fig. 9. Contractions of isolated guinea-pig's intestine in 10 c.c. bath. Contractions produced by acetylcholine (at *Ac*), KCl (at *K*), $BaCl_2$ (at *Ba*) and nicotine (at *Nic*) before (*a*), during (*b*) and after (*c*) benadryl, 1 in 1 million. (For details see text.)

in bicarbonate-free Tyrode's solution. Using similar conditions we have been unable to confirm his results (Table 2). In each experiment three control

samples were set up. One was extracted without incubation to give the acetylcholine content of the intestinal strips before incubation, and two were incubated without either the addition of histamine or barium chloride. From the acetylcholine content of these samples it can be seen (a) that synthesis of acetylcholine occurs in the intestinal strips during incubation, and (b) that the amounts synthesized vary in individual samples. For instance in Exp. 4, synthesis of acetylcholine during the 40 min. of incubation, amounted in the one sample to 1·4, but in the other to 5·2μg./g. On the other hand, the addition of either histamine or barium chloride did not increase the yield of acetylcholine on incubation. The $BaCl_2$ was added either every 10 min. in amounts of 1 mg./c.c. (col. 6) or once at the beginning of incubation in amounts of 4 mg./c.c. (Exp. 3, col. 7) or of 8 mg./c.c. (Exp. 5, col. 7). Histamine was added every 5 min. in amounts of 0·2 or 1μg./c.c. Similar amounts were used by Ambache in his experiments.

TABLE 2. Effect of histamine, potassium and barium ions on synthesis of acetylcholine in intestinal strips

μg. acetylcholine per g. tissue

| | | | | | Incubated with | | | | |
| | | | | | | | | Histamine | |
| | Not incubated | Incubated controls | | 3 mg./c.c. KCl | 5 mg./c.c. KCl | BaCl₂ | | 0·2 μg./ c.c. | 1 μg./c.c. |
Exp.	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
1	6·0	8·5	9·0	10·3	—	8·6	—	8·0	—
2	6·0	8·1	8·7	8·8	—	8·7	—	8·3	—
3	7·0	8·8	8·7	12·0	—	5·0	12·4	12·4	7·4
4	7·4	8·8	12·6	9·9	11·0	—	—	—	—
5	9·0	9·9	11·1	12·5	12·0	13·0	12·9	—	—

The effect of potassium ions on the synthesis of acetylcholine was also examined, since they are known to have an accelerating effect in slices or brei of brain tissue. It was found, however, that potassium was ineffective or accelerated the synthesis in one out of five experiments only. We must therefore conclude that the spontaneous synthesis of acetylcholine which occurs in incubated intestinal strips cannot be accelerated by changes in the incubation medium which might have an effect on tissue slices or brei. The difference between our results and those of Ambache, may be explained by the fact that he used only one control sample in each of the few experiments carried out at all, and that the effects of histamine and barium which he observed are accounted for by individual variations in the spontaneous synthesis.

DISCUSSION

The results of the present experiments do not support the conception that small doses of acetylcholine owe their stimulating effect on the small intestine to a nicotine-like action on the nerve cells in the intestinal wall, as stated by

Ambache. We could not confirm his results on which this statement is based, that the sensitivity of the intestine to acetylcholine is reduced during nicotine paralysis. It is true that the strong contractions produced by large doses of nicotine are sometimes followed by a period of reduced sensitivity of the muscle to acetylcholine, but this period is of short duration and the muscle kept paralysed by nicotine regains its original sensitivity to acetylcholine. In addition, during the period of reduced sensitivity to acetylcholine, the muscle is also less sensitive to other muscle-stimulating substances. The temporary depression is not a specific after-effect of a nicotine contraction but, according to Cantoni & Eastman (1946) follows any maximal contraction, except that produced by KCl. Ambache came to his conclusion because he failed to carry out two necessary controls: (a) testing drugs other than acetylcholine during nicotine paralysis, and (b) keeping the muscle paralysed long enough for the immediate after-effect of the strong nicotine stimulation to wear off.

Our results again do not support the view that the stimulating action on the intestine of barium and of potassium is due to a release of acetylcholine from nerve fibres originating from the plexus of Auerbach, and that this mechanism accounts for part of the stimulating effect of histamine. Ambache based his conclusions mainly on the assumption that cooling the intestine preparation for a few days inactivates the nervous structures in the intestinal wall, without impairing the sensitivity of the muscle fibre itself. Cooling the intestine for such periods, however, was found to reduce the sensitivity to direct muscle-stimulating drugs, and there is no significant difference between the reduction in sensitivity to either acetylcholine, pilocarpine or histamine. On the guinea-pig's intestine, but not on that of the rabbit, some evidence was found that cooling had a stronger effect on the response to potassium, and particularly to barium, than to the other drugs examined. It is difficult at present to explain this difference satisfactorily. The following facts, however, have to be taken into account. Since the dose-response curve varies with different substances it is difficult to compare quantitatively the effect of cooling on the response to various drugs. In addition, as far as KCl and particularly $BaCl_2$ are concerned, comparable responses are difficult to obtain even on the fresh preparation with repeated administration of the same dose of either. Nevertheless, we could confirm Ambache's observation that a condition of the cooled muscle can be obtained in which it is practically insensitive to large doses of $BaCl_2$, but responds to acetylcholine, at least when added in large doses. In this way $BaCl_2$ resembled nicotine to some extent, but no evidence could be obtained that $BaCl_2$ acted on the nerve cells in the intestinal wall. During nicotine paralysis, the preparation responded well to $BaCl_2$. This result in itself does not exclude a ganglionic action of $BaCl_2$ since it is known that nerve cells may be paralysed specifically to one but not to another drug. For instance, after curare the sympathetic ganglion cells become insensitive to acetylcholine, but

not to potassium (Brown & Feldberg, 1936a). If BaCl$_2$ were to act, however, like nicotine by stimulating the nerve cells in the intestinal wall, the effect, like that of nicotine, should be abolished by atropine. Atropine had no regular action of this kind.

The conclusion that BaCl$_2$—and the same applies to KCl and histamine—acts on nervous structures in the intestinal wall was based in addition on results obtained with eserine and on experiments concerning the synthesis of acetylcholine. Ambache had observed that eserine sensitizes the response to histamine, KCl and BaCl$_2$. It could be shown, however, that in the conditions of his experiments eserine had a similar action on the response to other drugs such as choline, 2268 F or acetylcholine. Ambache had omitted to show that the sensitization was specific. The effect of eserine can easily be explained as follows. Eserine, by its cholinesterase-inhibiting effect, leads to accumulation of acetylcholine, the effect of which sums with that of the substance to be tested on the intestine. In fact, under the conditions of Ambache's experiments, the accumulation of acetylcholine was so great as to produce contraction of the muscle by itself, so that the increased responses which he obtained with histamine, BaCl$_2$ and KCl was a combined effect of these substances with the accumulated acetylcholine. Even when the accumulation of acetylcholine is proceeding at a rate insufficient to stimulate the muscle by itself, it can still augment the response to any other stimulating drug. This condition was obtained in the present experiments when the effect of eserine was wearing off. The so-called eserine sensitization obtained by Ambache does not, therefore, supply evidence for an indirect mechanism of drug action. Nor could any evidence be obtained in favour of his theory by examining the responses to the various substances on the intestine paralysed by small doses of atropine or benadryl. The use of benadryl, in fact, enabled us to exclude conclusively the theory that a release of acetylcholine participates in the muscle-contracting effect of histamine, because with benadryl it is possible to abolish the response of the intestine to histamine without impairing that to acetylcholine, when it is either added to the bath or released from nervous structures in the intestine.

No evidence of an increased yield of acetylcholine on incubation of intestinal strips with either BaCl$_2$ or histamine could be found in our experiments. These results are contrary to those of Ambache who, however, carried out only a few experiments and did not make allowances for the individual variations which may occur in different samples of intestinal strips. His method of incubating relatively large pieces of intact intestinal tissue seems in fact unsuitable for studying the effect of substances, added to the incubation medium, on the synthesis of acetylcholine which occurs within the tissue. Even potassium, which is known to have an accelerating effect on the synthesis of acetylcholine, only occasionally affected the synthesis under these circumstances. It is possible that the substances added to the incubation medium are unable to

penetrate to the site of synthesis, when it occurs in pieces of intact intestinal tissue.

Therefore, the evidence given by Ambache for an indirect mechanism of action on the intestine of histamine, $BaCl_2$ and KCl cannot be accepted. This theory can certainly no longer be accepted for histamine. In the case of KCl, it is known that it can release and accelerate the synthesis of acetylcholine, but as far as its effects on structures other than the intestine are concerned, they are independent of the release of acetylcholine. It has been shown (Brown & Feldberg, 1936 *b*), for instance, that the stimulating effect of KCl on sympathetic ganglia is independent of the acetylcholine metabolism, because it occurs also on the denervated ganglion which contains no acetylcholine. There is at present no evidence that a release of acetylcholine by KCl contributes to its muscle-stimulating effect on the intestine; if such a mechanism were involved in the muscular contraction observed after KCl its contribution could be small only, and it would be difficult to detect it without being able to abolish the direct stimulating action of KCl.

The response to $BaCl_2$ was certainly affected by cooling the guinea-pig's intestine to a greater extent than that to other drugs. This discrepancy between $BaCl_2$ and other drugs, however, was not observed on the cooled rabbit's intestine. It is unlikely that the mode of action of $BaCl_2$ is fundamentally different in the two preparations. Before the theory that $BaCl_2$ owes its stimulating action on the gut to the release of acetylcholine can be accepted it is necessary to show that it can in fact release acetylcholine from perfused tissues containing cholinergic nerve fibres. At the present stage of our knowledge we are not justified in attributing such an effect to $BaCl_2$, and it seems rather dangerous to interpret the changes which occur in the sensitivity of the intestine to drugs after cooling solely to inactivation of nervous elements, since cooling impairs the muscular tissue as well. Similarly, in our opinion, it is not possible to postulate a nervous origin for the rhythmic contractions of the longitudinal muscle of the intestine simply because they disappear in the cooled preparation.

SUMMARY

1. No evidence could be obtained in support of the theory that small doses of acetylcholine owe their muscle-contracting property on the isolated intestinal preparation to a nicotine-like effect on ganglion cells. Paralysing doses of nicotine were not found to affect the sensitivity of the preparation to small doses of acetylcholine, as was stated by Ambache. It is true that in some preparations the strong contraction, which is produced by a large dose of nicotine and precedes the stage of paralysis, renders the preparation less sensitive to acetylcholine. But in this condition the preparation is also less sensitive to other muscle-stimulating drugs; this condition lasts for a short period and is independent of the nicotine paralysis.

2. No evidence could be obtained in support of the theory that the muscle-contracting effects of $BaCl_2$ and KCl on the isolated intestine result from a release of acetylcholine from the nervous structures in the intestinal wall and that such a mechanism accounts also for part of the contraction produced by histamine. This theory of Ambache was based on the assumption that cooling an intestinal preparation inactivated only the nervous structures of the preparation. We have found, however, that the treatment also impaired the muscle fibres.

3. The theory of Ambache was supported by experiments which were meant to show that $BaCl_2$ and histamine increase the yield of acetylcholine in incubated strips of intestinal tissue, and that eserine sensitized the preparation to these substances. No evidence could be found for an accelerating effect of $BaCl_2$ or histamine on the synthesis of acetylcholine under the conditions used by Ambache, and his results can be explained by the fact that no allowance was made for the great individual variations which occur in different samples of incubated intestinal strips. The sensitizing effect of eserine observed under the conditions of Ambache's experiments was found to occur with all muscle-stimulating substances and is explained as follows. Eserine by its cholinesterase-inhibiting action leads to the accumulation in the intestinal wall of acetylcholine, the effect of which sums with that of any muscle-stimulating substance added in this condition.

4. When histamine, $BaCl_2$ and KCl were examined on the intestinal preparation paralysed by small doses of atropine or by benadryl no evidence could be obtained in favour of Ambache's theory.

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PROCEEDINGS

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Determination of glomerular filtration rate, renal plasma flow and tubular mass in man by multiple injection technique. By J. A. BARCLAY, W. T. COOKE and R. A. KENNEY

TECHNIQUE

Glomerular filtration rate and renal plasma flow. With the subject recumbent, 15 c.c. blood (for blank determinations) are withdrawn from a vein and 40 c.c. 10% mannitol solution given in 1-2 min. through the same needle. Following preliminary injection of 2% procaine into axilla in the anterior axillary line, 6 c.c. 35% diodone (or 4 c.c. 20% para-amino-hippuric acid) made up to 20 c.c. with 10% mannitol are injected slowly into the subcutaneous tissues. To ensure adequate urine flow, 600-900 c.c. water are drunk by the subject.

25 min. after diodone injection, the bladder is completely emptied and urine discarded. The initial blood sample (15-20 c.c.) is withdrawn. Thereafter, urine samples are collected (where possible without catheterization), timed, measured and saved every 15-20 min. and blood samples every 30-40 min. depending upon the duration of the test.

Tm diodone. Immediately on collection of last urine specimen, a blood sample is withdrawn and 20 c.c. 35% diodone rapidly injected intravenously. A blood sample is taken 15 min. after this injection and urine is collected 15 min. later.

ANALYTICAL METHODS

Diodone is determined by the method of Barclay & Kenney (1945).

Para-amino-hippuric acid is determined by Goldring & Chasis's (1944) modification of the Marshall & Bratton's procedure with an alteration in that a 5 c.c. sample is used instead of 10 c.c.

Mannitol is estimated by a method based on that of Voris, Ellis & Maynard (1940). 2 c.c. of the sample are taken in a boiling-tube and to it are added 1 c.c. of a 0·2% solution of sodium periodate and 5 c.c. of Sorenson phosphate buffer at pH 7. The solutions are mixed and allowed to stand at room temperature for 15 min. The residual periodate is titrated with N/20 sodium

thiosulphate from a Conway micro-burette, using as indicator a solution of starch screened with phenol red. Standards are titrated along with each set of samples.

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Synthesis and accumulation of acetylcholine at motor end plates.

By R. J. S. McDowall and A. Z. Shafei. *Department of Physiology, King's College, London*

The liberation of acetylcholine at motor nerve endings is now well recognized. We have now demonstrated that conditions which reduce or increase the synthesis of acetylcholine in brain tissue and brain extracts affect in a similar way the responses to nervous stimulation of the phrenic diaphragm preparation of Bülbring (1946) suspended in various modifications of Tyrode's solution. Thus they are decreased by calcium and glucose and are increased when calcium and glucose are reduced. The response to direct muscle stimulation is not so much affected and is not always parallel. It is however present after curare.

An accumulation of acetylcholine at resting nerve endings is suggested by the occurrence, after a period of rest, of a few supernormal contractions with delayed relaxation at the beginning of a series of nerve stimulations, but this is less evident when the muscle is stimulated directly after a similar rest. These results are made still more evident by eserine which prevents the destruction of acetylcholine especially when glucose is absent and calcium deficient.

It is also found that nerve stimulation may reduce or, if sufficiently long, abolish the twitches produced by eserine, which, as suggested by Feldberg (1945) may be due to such an accumulation. Here we may consider that nerve stimulation reduces the accumulation. On the other hand, the eserine twitches are abolished by calcium (Langley & Kato, 1914). This may be looked upon as an inhibition of synthesis. Similarly, since the twitches in tetany are also abolished by calcium, it would seem that the blood calcium normally inhibits excessive synthesis.

As the muscle recovers from the effects of cooling which abolishes nervous action, its responses to direct stimulation, which have been previously quite regular, become irregular as soon as nerve action appears, and this irregularity may at first be abolished by nerve stimulation and by calcium and glucose. It is therefore suggested that the irregularity is due to the variable amounts of acetylcholine accumulating at individual nerve endings and varying their excitability. The recognition of a resting variable activity of nerve endings would also explain why an increased accumulation of acetylcholine in the

presence of eserine produces twitches and not an increased tone and possibly also the occurrence of rhythmical changes of potential in eserinized and denervated muscle (Brown, 1937).

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Application of adenosine triphosphate and related compounds to the spinal cord of the cat. By FRITZ BUCHTHAL, LISE ENGBAEK, O. STEN-KNUDSEN and E. THOMASEN. *Institute of Neurophysiology, University of Copenhagen*

Direct application to the isolated fibre and close arterial injection of the total muscle by adenosine triphosphate (ATP) stimulates normal and curarized frog and mammalian muscle (Buchthal, Deutsch & Knappeis, 1944; Buchthal & Kahlson, 1944). ATP also sensitizes muscle to acetyl choline (Buchthal & Folkov, 1944; Buchthal & Kahlson, 1944).

In order to investigate a possible stimulating effect of energy-rich phosphate compounds on the anterior horn cells, we have developed a method of close arterial injection to the cervical segments of the spinal cord of chloralosed cats. The substances are injected in iso-osmotic solutions, generally in a volume of 0·3 ml. One vertebral artery was cannulated before its entrance into the transverse foramen and both arteries were cut off in the transverse process of the atlas by drilling a hole until it bled profusely and then closing it up with wax. During the injections the non-cannulated vertebral artery was temporarily blocked in its lower part. Injection of indian ink showed the localization of the substances to the cervical segments. Action potentials in different muscles of the upper extremity were recorded simultaneously as an index of the stimulating effect on the anterior horn cells.

Na-ATP prepared from the Ba salt or the Ca salt in concentrations of $5\text{-}25 \times 10^{-6}$ mol./ml. evokes strong tetanus-like contractions in the muscles of the upper extremities. This corresponds approximately to an amount of $200\text{-}1000\,\mu g$. ATP/g. tissue, the weight of the injected region being about 3 g. The discharges, which start during the injection, continue for several seconds, and show a pattern of asynchronous activity of different motor units even in the same muscle. The threshold concentration is about 5×10^{-6} mol./ml.

Inorganic triphosphate and pyrophosphate likewise evoke contractions when applied in equimolar concentrations with ATP. Creatine phosphate in equimolar concentration is without effect and only causes contractions in amounts

thirty times larger than the threshold dose for ATP. Muscle adenylic acid, in amounts up to five times the threshold dose for ATP, is also without effect. Both these substances stimulate the cells of the perfused sympathetic ganglion (Feldberg & Hebb, 1946). Sodium orthophosphate in equimolar concentrations up to five times the threshold amounts for ATP, and acetylcholine in small and large quantities (1–100 μg.) likewise have no stimulating action on the anterior horn cells of the spinal cord.

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A simple micro-stimulator. By H. HARTRIDGE. *Physiological Laboratory, St Bartholomew's*

If a student's microscope with a 1/6th-in. objective is available, a simple micro-stimulator can be made with a little additional apparatus, in the following manner:

A wooden box is obtained, having two sides roughly 40–50 cm. apart. A circular hole is cut in the middle of one of these sides, having a diameter equal to the distance between the feet of the microscope. The box is placed on its side, and the microscope placed over the hole. When the eyepiece is removed, it will be found that the objective produces a reduced image of objects placed inside the box.

An ordinary piece of silvered plate-glass may be placed over the draw-tube of the microscope, so as to deflect the rays from the objective horizontally into the observer's eye. A silvered-surface mirror gives better results, and a right-angled glass prism better results still.

It will be found that there is right and left transposition, between the objects placed in the box and the images of those objects seen through the microscope and prism. If this is a disadvantage, it may be corrected by placing an ordinary erecting prism at some suitable place on the path of the beam of light between the objective and the eye of the observer. The total distance between objective and observer, via the prism, should be about 50 cm.

Now if the objective be 4 mm. focal length, the constants of this apparatus will be found to be a field diameter of approximately 60 cone units, each unit being equal to 1 cm. Thus an object 5 cm. long, placed inside the box, should have a length corresponding to 5 cone units.

The above arrangement will be found to work quite well, but does not of course produce images of the same definition as those given by an instrument which has been specially designed for the purpose.

Chromatic difference of magnification of the eye. By H. HARTRIDGE. *Physiological Laboratory, St Bartholomew's*

When light passes through the eye media in order to approach the retina, it suffers two kinds of dispersion simultaneously: lenticular and prismatic. The lenticular dispersion produces chromatic dispersion of focus. The prismatic

dispersion which produces chromatic difference of magnification is caused by the difference in position of the fovea and the axial point. Both are serious obstacles which have to be dealt with when attempting to apply micro-stimuli to the human retina.

It is, unfortunately, impossible to deal effectively with chromatic difference of focus by means of correcting lenses, because these introduce chromatic difference of magnification of their own. Two methods have therefore been adopted in order to overcome the effects of chromatic difference of focus so far as possible: (1) to avoid the use of coloured lights which differ greatly in wave-length; where this is not possible, (2) to place the sources at different distances, so that the images of both are sharply focused at the same time on the retina.

Micro-stimulation has been found to provide a relatively simple method, both for measuring and for correcting the chromatic difference of magnification of the eye. Measurement has been effected by placing three test sources apparently into line with one another, the two outside ones which were 6 cone units apart being red, the middle one being blue-green. It was found that when they appeared to be in line, in fact they were out of line in the visual field.

The red sources had to be displaced 0·48 cone unit to the left of the blue-green one, and 0·70 cone unit vertically below it, for all three to appear in line to the observer's left fovea, both horizontally and vertically.

Having measured the chromatic difference of magnification in this way, it was found that it could be corrected very simply by decentring a convex lens in front of the observer's eye. If this decentration is of precisely the right amount, there is a very near correction for chromatic difference of magnification, both horizontally and vertically.

A convex lens of 1·25 D, decentred vertically downwards by 11·3 mm. and to the left by 7·8 mm., was found to correct the chromatic difference of magnification of the author's left eye at the fovea.

Since the amount of chromatic difference of magnification depends on the position of the fixation point relative to the axial point, and since this varies in different eyes, the former will vary also. It will therefore be necessary to measure and to correct each eye separately.

Some observations on the retinal image of a point source.

By H. HARTRIDGE. *Physiological Department, St Bartholomew's*

By means of the apparatus described in the accompanying communication, the retina of the author's left eye was stimulated by narrow beams both of monochromatic and of white light.

It was found that the visible image of a point source with a pupil of 4 mm. diameter is approximately the same size as a foveal cone. This fact was ascertained by adjusting the distance between two such sources until the intervening dark space between their images appeared to be equal in width to the diameters of the images themselves. This small size of the retinal image fits in with the high acuity which is observed both with white and with coloured lights, and makes the employment of retinal inter-connexions, such as Polyak has proposed, unlikely so far as the centre of the human fovea is concerned.

The visual acuity, in sunlight, of the author's left eye, when chromatic dispersion has been corrected, is such that all the letters in the 5 m. line of Hay's test type and half the letters in the 4 m. line can be read correctly at a distance of 10 m. The author's right eye has inferior acuity to this, owing to irregular refraction of the crystalline lens; it is for this reason that the left eye is used almost exclusively for visual tests.

Tests were done under two different conditions: (1) out-of-doors in bright sunlight, silver-paper lines, 1 mm. wide and 12 mm. apart, being placed on a black background; (2) in a room lit by ordinary daylight, the micro-stimulator being used, the test objects being two point sources of white light obtained by employing two 3½ V. lamps run from a dry battery. The eye was light-adapted. No precise adjustment of illumination was found to be required. As stated above, the pupil was about 4 mm. in diameter.

All the receptors lying in the retinal area under investigation were found to have approximately the same thresholds as one another at different parts of the visible spectrum.

Colour appreciation was found to be deficient with these small light stimuli. When a black background was used, yellow was usually replaced by white, and blue by slate-grey; red appeared orange-brown; while blue-green was much diluted with white. In spite of these changes, it was usually possible with a little practice to identify test-objects of the three primary colours: red, green and blue. When, on the contrary, a white background was in use, all colours tended to be replaced, or were actually replaced, by shades of grey, according to their size, hue and intensity, as would be expected from the observations made previously on the anti-chromatic responses.

The high degree of acuity observed during these experiments, the exclusive use of the fovea for purposes of observation, and the preservation of light adaptation, all make it unlikely that rods were being stimulated accidentally instead of cones.

Colour-blindness at small visual angles. By H. HARTRIDGE.
Physiological Department, St Bartholomew's

It will be remembered that a form of reduced vision is obtained when test-sources subtending small angles at the eye are being examined by an observer with normal colour vision. This reduced vision superficially resembles a form of dichromatic vision, where orange-red is one primary and a greenish blue is the other. Thus Willmer and Wright have shown that when red of 6500 A. and blue of 4600 A. are mixed together, all parts of the spectrum may be matched, there being two neutral points, one at 5800 A. and the other at about 4100 A.

It was found by the author, that a further reduction of visual angle produced what has been called 'colourless foveal vision'. The red and orange of the spectrum become neutral tint—usually dark-grey or black. The blue-green part of the spectrum alters also and becomes pale green or white, and is then identical, or nearly identical, with the yellow part of the spectrum which had previously become white. Under these circumstances the whole of the spectrum is seen to consist of neutral tints.

There seems to be no doubt that this 'colourless' vision is obtained in the fovea in a region which is stated to be devoid of rod receptors. The supposition is advanced that this colourless vision, like the reduced colour vision previously described, is due to the antichromatic responses. These mechanisms usually act in the following order: if at first there is colourless foveal vision, an increase of visual angle causes the 'red' mechanism to come into play; a slight increase in angle causes the 'blue-green' mechanism to act. At this stage there is reduced, that is, dichromatic, colour vision. As the visual angle is further increased, first the 'yellow' mechanism and then the 'blue' mechanism come into play, thus restoring full colour vision.

The above experiments were done with the fully light-adapted eye, the pupil being of approximately 4 mm. diameter, and the test source 0·1 cone unit in diameter. As the intensity of illumination of this source was varied by means of neutral tint filters, all the changes took place between full trichromatic vision, reduced colour vision, and colourless foveal vision.

Different fixation points for lights of different colour.
By H. HARTRIDGE. *Physiological Department, St Bartholomew's*

Two methods have now been used for determining the relative positions of the fixation points of the author's left eye. The first depended on the measurement of the eye movements which were required in order to fix different coloured test objects in turn. This method gave the result that the green fixation point lies

near the apex of an equilateral triangle, the red fixation point being to the right and the blue fixation point to the left of the base of this triangle. The second method made use of the fact that there is situated, close to the fixation area of the author's left eye, a small scotoma for red rays, the position of which could be identified without any special difficulty when using the micro-stimulation apparatus. Details of the method are, that having fixed a light of given wavelength, produced by a spectro-illuminator, the position of the small blind-spot for red rays was ascertained by means of a movable red test source. The results confirmed those given by the previous method.

The fixation points for red, orange, yellow, yellow-green, and green were found to lie approximately on a straight line which sloped downwards to the left; and the fixation points for green, blue-green, and blue also fell approximately along a straight line which sloped downwards, to the right. The red and blue points occupied positions which were almost identical with those previously obtained. The points for the other colours occupied intermediate positions.

This result fits in with the view that there are more than three varieties of colour receptor in the human fovea. It is also in agreement with the Cluster hypothesis.

If in fact the retinal receptors of different response are arranged in the fovea in a haphazard manner, it is unlikely that the fixation points will occupy precisely the same positions in any two eyes. But the methods outlined above, particularly the first, should enable their positions to be ascertained.

The response of the yellow receptor of the human retina.

By H. HARTRIDGE. *Physiological Laboratory, St Bartholomew's*

It has been found possible to obtain an approximate idea of the shape and spread of the response curve of one of the receptors responsible for human vision, namely, that in the yellow, which has a maximum at the wave-length of 5850 A. The form of the curve was obtained in the following way: as the visual angle of a yellow test object is reduced progressively in size, an angle is reached at which it is replaced by white. If now a yellow object is placed near it, it reverts once more to yellow. By using objects of other colours than yellow, their effectiveness in causing the replacement of yellow is a measure of their ability to stimulate the receptor responsible for the phenomenon.

In order to make the test, two sources were presented to the eye of the observer by means of a micro-stimulator. One of these was a small electric lamp, over which was placed a yellow colour-filter—this was the test-source. The second source was a beam of monochromatic light, produced by a constant-deviation spectroscope from a source of uniform energy content. The intensity of the latter could be varied by inserting Ilford standard neutral-tint filters.

Measurements were performed by ascertaining the wave-length at which the test-source changed from yellow to white, or vice versa. The values obtained are shown in Table 1. When these values are plotted, it is found that the shape of the curve obtained resembles those found by Granit in the retinae of many mammals.

TABLE 1

Log of intensity	A.	
− 5	5850	
− 4	6030	5680
− 3	6110	5560
− 2	6240	5390
− 1	—	—
0	—	—

Three conclusions appear justifiable: the response curve belongs to a receptor which is definitely in the yellow part of the spectrum, that is, it could not be identified with the wave-lengths of the hypothetical response curves for the red and green sensations on Young's theory of vision. In the second place, the curve resembles those obtained by Granit, in its general configuration. And thirdly, so far as can be judged, its spread is very similar to those of the receptors discovered by Granit.

Many observers have found that, using mixtures of three monochromatic primaries, it was not possible to match precisely all parts of the visible spectrum. In the author's opinion the time has come to see if mixtures of four primaries will not enable precise matches to be obtained. The wave-lengths of these primaries might be: 6500, 5800, 5000, and 4500 A.

The yellow human receptor by another method. By H. HARTRIDGE.

Physiological Department, St Bartholomew's Hospital Medical College, London, E.C. 1

Wright showed, in 1943, that when the area of human fovea investigated is small, the luminosity curve of the spectrum alters somewhat in shape, particularly on the long wave-length side (Fig. 1, *A, B*).

When a yellow test object subtends a sufficiently small angle at the eye, it loses its colour and is replaced by pale grey, or white. When a blue test object is similarly treated, it also loses its colour and becomes dark grey, or black. If the loss of colour is due to the yellow and blue receptors respectively ceasing to send their responses to the brain, there should be a difference in the luminosity curves for large and for small retinal areas in two regions, namely, those occupied by the yellow and by the blue receptors respectively. In the author's opinion curve *C* (Fig. 1) shows the responses at different wave-lengths of the yellow receptor, and similarly curve *D* those of the blue one.

In Fig. 2, curve *C* is the same as curve *C* in Fig. 1, but increased in height; curve *E* is the yellow response curve obtained by the first method, described

in a previous communication; curve F is the response curve of a yellow receptor obtained by Prof. Granit in the cat, using his selective adaptation technique. These three curves are similar in the following respects: their maxima are very close in wave-length; they resemble one another in general
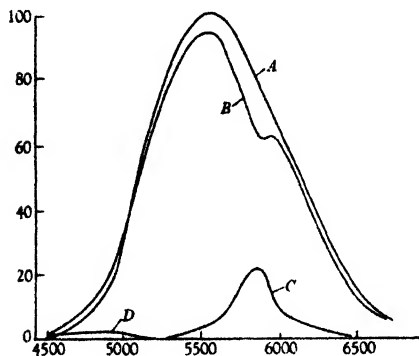

Fig. 1.   Fig. 2.

Fig. 1. Luminosity curve: A for large retinal areas; B when area of fovea subtends 20 min. of arc. Subtracting B from A, after making values at 5100 equal, gives C and D. Abscissae = wavelength. Ordinates = arbitrary intensity units.
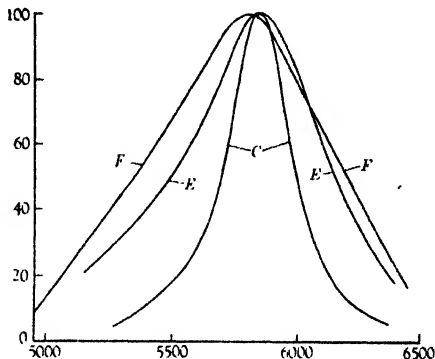
Fig. 2. Abscissae = wavelengths. Ordinates for curves C and E = arbitrary intensity units. Ordinates for curve F = percentage nerve responses.

shape. Individual differences can probably be accounted for by variations in the intensity and spectral purity of the monochromatic radiations employed by the three experimenters—Prof. Granit (curve F), Dr Wright (curve C) and the author (curve E). Evidence seems to be slowly accumulating in favour of the view that human vision is not trichromatic as has long been supposed.

The action of acetylcholine on sensory endings. By J. A. B. GRAY. *From the National Institute for Medical Research, N.W. 3*

Records have been taken with amplifier and oscillograph from the peripheral end of a cutaneous nerve during arterial injections into the skin of acetylcholine (10 mg. of acetylcholine in 1 ml. of 5% NaH_2PO_4 diluted to 100 ml. with Locke's solution). In five out of five cats and in three out of four dogs injection of this acetylcholine solution set up a discharge of fast nerve impulses, lasting for several seconds after the end of the injection. In some preparations, the control solution set up a discharge during the actual injection, but this never persisted. Solutions of acetylcholine in one-tenth the strength behaved like controls. Some of these results have been obtained in preparations in which a flap of skin remained attached only by a pedicle containing artery, vein and nerve and also in animals which had received 1 mg. of atropine sulphate intravenously.

Coon & Rothman (1940) showed that a nicotine-like action of acetylcholine could produce a pilomotor reflex, abolished by sympathectomy. My results were not secondary to this reflex, since they could be obtained in a cat three weeks after sympathectomy.

Responses like those from the skin were obtained when acetylcholine was injected into an arterial arch of the mesentery and records were taken from the corresponding mesenteric nerve. A preparation was chosen in which there were a number of Pacinian corpuscles. According to Gernandt & Zotterman (1946) fast fibre responses of the type obtained could only have come from Pacinian corpuscles, and the 'pressure' responses during injections support this view, since Pacinian corpuscles are known to be sensitive to changes in arterial pressure (Gammons & Bronk, 1935).

Since nerves in continuity are not excited by acetylcholine it is concluded that some sensory endings and Pacinian corpuscles in particular are sensitive to acetylcholine. This suggests that sensory endings, like the central ends of post-ganglionic fibres and motor end-plates, may be specialized to facilitate the starting of propagated responses.

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Ribonucleic acid as a constituent of labile liver cytoplasm. By
Rosa M. Campbell and H. W. Kosterlitz. *Physiology Department, Marischal College, Aberdeen*

It has been suggested (Kosterlitz, 1944) that ribonucleic acid and not deoxyribonucleic acid accounts for the loss of total nucleic acid from the liver when rats, previously fed on a diet adequate in protein, are given a protein-free diet. During fasting, ribonucleic acid is lost from the liver while its deoxyribonucleic acid content remains unchanged (Davidson & Waymouth, 1944; Davidson, 1945).

The deoxyribonucleic acid content of liver tissue, calculated for 100 g. of initial body weight, has now been found not to be influenced by the protein content of the diet (Table 1). The concentration of deoxyribonucleic acid, however, was higher in (male) rats fed on a protein-free diet than in those fed on an 18% casein diet. If the results obtained for the rats fed on the protein-free diet are recalculated for 0·213 g. deoxyribonucleic acid—the value found in the rats fed on the 18% casein diet—then a direct comparison may be made between the two groups of rats. Feeding a protein-free diet for one week caused losses of protein, phospholipin and ribonucleic acid amounting to 40,

TABLE 1

Diet (number of rats)	Water	Glycogen	Neutral lipids	Protein	Phospho-lipin	Ribo-nucleic acid	Deoxyribo-nucleic acid	Deoxyribo-nucleic acid
			g./100 g. fresh liver					mg./100 g. initial body weight
18% Casein (4)	69·05 ± 0·10	3·40 ±0·49	1·63 ±0·03 (3)	16·4 ± 0·4	3·09 ±0·02 (3)	1·01 ±0·02	0·213 ±0·010	8·05 ±0·16
Protein-free (4)	70·3 ± 0·25	7·21 ±0·46	2·37 ±0·09 (3)	12·1 ± 0·2	2·08 ±0·01 (3)	0·82 ±0·02	0·263 ±0·005	7·9 ±0 20
			g./0·213 g. deoxyribonucleic acid					
Protein-free (4)	56·9 ± 0·95	5·84 ±0·46	1·91 ±0·05 (3)	9·8 ±0·2	1·68 ±0·06 (3)	0·66 ±0·02	0·213	,

46 and 35% respectively, the smaller loss in ribonucleic acid being possibly due to its presence in the nucleoli. The water content of the liver decreased by 18% and its glycogen and neutral lipid contents increased by 72 and 17% respectively. Ribonucleic and deoxyribonucleic acids were estimated by a modification of the method of Schmidt & Thannhauser (1945).

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On the heat production in human muscle during voluntary contraction. By H. BARCROFT and J. L. E. MILLEN

Lippross (1942) and Buchthal, Høncke & Lindhard (1944) found that when the circulation to human muscle was arrested, activity caused a rise in temperature due to chemical heat production. Barcroft & Millen (1939) observed that the circulation in the gastrocnemius soleus was arrested during tiptoe standing on one leg. In the following experiments relative changes in the temperature of the gastrocnemius soleus were recorded, to the nearest 0·05° C.

Exp.	Subject	Duration of tiptoe stand (min.)	Rise in muscle temp. (° C.)
1	J.L.E.M.	2·5	0·35
		2·5	0·35a
		3·25	0·50a
		2·75	0·40
2	J.L.E.M.	2·75	0·35a
3	H.B.	3·0	0·35a
4	H.B.	3·25	0·40
		2·87	0·40

by a thermojunction deeply embedded in it (Barcroft & Millen). The leg was in water at 37° and before each tiptoe stand it was rested until the temperature remained constant within 0·05° C. over a period of 20 min. In all cases tiptoe standing, in the bath, was continued for as long as possible. In some the circulation in the thigh was arrested a few minutes before standing (*a*).

Errors due to heat gain or loss were small since muscle temperature was almost constant before standing, and remained constant afterwards when the circulation was arrested in the thigh (Fig. 1).
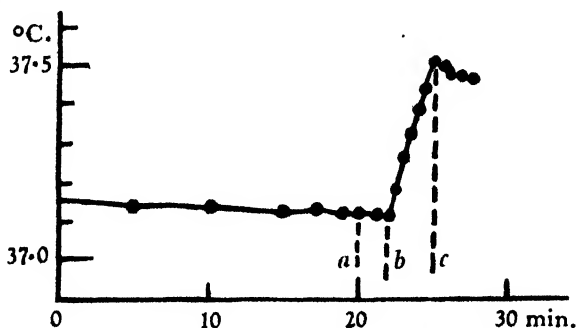
Fig. 1. *a*, circulatory arrest; *b*, stand on tiptoe; *c*, sit down.

Taking the specific heat of muscle as 0·85 (0·88 for frog muscle (Hill, 1931)) the heat production during a voluntary contraction maintained for as long as possible was $0·85 \times 0·4 = 0·34$ cal./g. muscle. Peters (1914) obtained 0·9 cal./g. frog muscle in air indirectly stimulated till fatigued.

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Pharmacologically active substances in the fluid of nettle hairs (*Urtica urens*). By N. EMMELIN and W. FELDBERG.* *Physiological Laboratory, Cambridge*

The fluid in the hairs of the nettle contains at least three substances which contract smooth muscle. One of these is acetylcholine, another is probably histamine and the third has not yet been identified.

Acetylcholine. When a single hair of a nettle is brought into a 10 c.c. bath in which is suspended a piece of the guinea-pig's or rabbit's intestine, the muscle contracts. The substance responsible for this effect is alkali-sensitive and destroyed by cholinesterase but not in the presence of eserine. It has been identified pharmacologically as acetylcholine. Each hair from the stalk of the

plant contains between 0·02 and 0·4 μg., usually between 0·08 and 0·12 μg., of acetylcholine. As the fluid content of the hairs is not more than 8 μg. the concentration of acetylcholine must be of the order of 1 in 100 or stronger. The hairs from the leaves are much smaller and contain on the average 0·03 μg. acetylcholine.

Histamine. When the content of 15–30 hairs is tested after atropine either on histamine sensitive smooth muscle preparations or on the arterial blood pressure of the cat the presence of histamine (or of a histamine-like substance) can be detected. Its concentration in the hair fluid is about 1 in 1000.

Unidentified substance. When the content of 150–300 hairs is tested after atropine on the rabbit's intestine preparation, the muscle contracts slowly. 300 hairs contain about 3 μg. histamine which would not affect the rabbit's intestine since it responds only to doses 50–100 times greater. The effect is therefore not due to histamine.

Mechanism of nettle sting. The histamine concentration in the fluid of the nettle hair is sufficiently strong to account for the triple response produced by the nettle sting. There is no necessity to assume a release of histamine from the skin itself in order to explain the similarity of histamine and nettle-sting reactions. Histamine 1 in 1000 pricked into the human skin produces itching but not the burning sensation characteristic of the nettle sting. In the presence of acetylcholine, 1 in 100, the histamine itching assumes a more burning character, but the sensation is usually not as strong as that produced with the nettle hair.

The effect of adrenaline upon striated muscle. By G. L. Brown, Edith Bülbring and B. Delisle Burns. *From the National Institute for Medical Research, N.W. 3*

We have used the isolated phrenic-diaphragm preparation of the rat (Bülbring, 1946) to study the effects of adrenaline upon the tension and action potential of striated muscle. As is well known, adrenaline (0·5–2 μg./c.c. bath fluid) has little effect upon the peak twitch tension of the unfatigued muscle excited with maximal nerve volleys. When the muscle is fatigued, adrenaline causes an increase in twitch tension if the muscle is excited through its nerve, but not if it is stimulated directly. Optical, isometric tension records, show that adrenaline can produce an increase in the duration of the twitch tension which is evident whether the peak tension is increased or not.

Electrical records show that adrenaline also produces an increase in the duration of the action potential which can be demonstrated whatever the circumstances of stimulation and whether the muscle is fatigued or not.

Records from directly stimulated, curarized single muscle fibres show that the increased duration of the action potential is probably due to a slower spread of excitation along the fibre. The effects of adrenaline can, in fact, be closely imitated by cooling the preparation from 38° to 35° C.

In a fatigued nerve-muscle preparation the synchronization of excitation between the fibres is less perfect than in a fresh muscle or one stimulated directly. It appears that adrenaline augments peak twitch tension only when the muscle units are contracting relatively asynchronously. We have no evidence that the increase in twitch tension which adrenaline evokes in a fatigued nerve-muscle preparation is due to an increase in the number of muscle fibres contracting. The direct effects of adrenaline upon the muscle fibre are, in our opinion, probably sufficient to account for its action.

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A class demonstration of the alpha rhythm. By E. D. ADRIAN

When demonstrations have to be made to large classes it is often convenient to present the data in terms of sound. The α-rhythm of the electro-encephalo-gram is too low in frequency to give a continuous note, but various methods can be used to make it audible. Adrian & Matthews (1934) employed a moving-iron loudspeaker with the diaphragm very close to the pole pieces; Bevers (1942) and Ross have used the α-wave potentials to change the pitch of a sound from an oscillating valve circuit.

The present method was originally developed for demonstrations on the heart rate. At Mr Hodgkin's suggestion a diode valve was introduced into the conventional amplifier and loudspeaker system to cut out all but the peak of the R wave; this could then be made audible without interference from the respiratory muscles. The same arrangement, with greater amplification, will make the waves of the α-rhythm audible as individual pulsations. The diode valve (Mullard EA 50) and its batteries form a separate unit which can be plugged in between the input amplifier and the loudspeaker unit. The volume control of the input unit is set so that the α-wave potential swing is about 8 V. and the diode is biassed so that only positive potentials of more than 3 V. are allowed to pass. The same method can be used to detect flicker potentials of low frequency.

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The antichromatic responses. By H. HARTRIDGE.
St Bartholomew's Medical College

When the angle subtended at the eye by isolated yellow or blue objects is sufficiently reduced, they are seen to lose their colour and to be replaced by shades of grey. This process of colour suppression is much more effective if the yellow and blue objects are situated side by side in pairs. Specially effective is it if the objects are caused to resemble as closely as possible, in width, tint and distribution, the fringes which would be produced by chromatic aberration.

The chromatic aberration of the eye may either be reduced or increased by suitable lenses placed external to the eye. When it has been doubled the colour fringes are still not noticeable, the illumination on the test objects being about 100 f.c., and the pupil about 4 mm. diameter. When it has been quadrupled, then the fringes are clearly visible. With a threefold increase the fringes are usually just observable. At greater light intensities the surplus correction is somewhat less, and at lower intensities it is somewhat greater than that found at 100 f.c. The conclusion is that the colour elimination suffered by blue and yellow is usually more than sufficient to correct the chromatic aberration present in a normal eye. It is this surplus correction which enables an observer to employ uncolour-corrected spectacles, hand magnifiers and eyepieces.

Now that the elimination of the fringes of chromatic aberration has been accounted for by the loss of colour suffered by blue and yellow, the latter in its turn requires an explanation. The phenomenon is not limited to the foveal centre, as has been supposed by Willmer and Wright, but has been found by the author to take place at all parts of the retina. The following tentative hypothesis has been advanced: that situated on the visual nervous pathways there are mechanisms which modify the central connexions of the retinal receptors. Adherents of the trichromatic theory may suppose that the synaptic connexions between the blue receptors and the blue visual centres are severed, thus causing blue to be replaced by dark grey or black; and that a synaptic connexion is effected between the green receptors and the blue visual centres, thus causing yellow, which normally sends nerve impulses to

the red and the green visual centres only, to send them to the blue visual centre as well, thus imitating the effects normally produced by white and by neutral colours, and so causing yellow to appear pale grey or white.

Those who suppose that there are specific yellow receptors, in addition to those of other colours, may adopt a somewhat simpler plan: that blue is replaced by dark grey or black by severing the synaptic path between the blue receptors and the blue brain centre, and that similarly yellow is replaced by pale grey or white by severing the synaptic path between the yellow receptors and the yellow brain centre. Evidence is slowly accumulating in favour of the latter hypothesis.

The phenomenon of precise colour matching by the mixture of three stimuli. By W. D. WRIGHT. *Imperial College of Science and Technology*

Experiment shows that, in general, if a radiation C is allowed to illuminate one-half of a photometric field of $2°$ angular subtense, it can be accurately matched both in colour and brightness by a suitable combination of only three radiations [usually chosen to be saturated red (R), green (G) and blue (B) lights], when mixed additively by a suitable optical device. Such a device might consist of a rotating prism system, an integrating sphere, the projection of the three beams on to the same diffusing surface, a rotating disk, and so on. If the amounts of the three stimuli required for a match are represented by r, g and b measured in some convenient units, the colour match can be expressed algebraically in terms of the equation

$$C = r.R + g.G + b.B.$$

However, if C happens to be, say, a monochromatic radiation taken from the blue-green part of the spectrum, it will be found that no positive combination of the red, green and blue matching stimuli will lead to an accurate match of C. Even when the intensity of the red stimulus is reduced to zero, the mixture of the blue and green stimuli will still give rise to a colour sensation which is always whiter or less saturated than that due to the monochromatic blue-green radiation. To allow for this, the matching stimulus R has to be mixed with the test stimulus C, and when this is done it is found that adjustment of the amounts of R, G and B will lead to an exact match between C plus R in the one half of the field, and G plus B in the other (for the example where C is a blue-green radiation). This match can be represented by the equation

$$C + r.R = g.G + b.B,$$

so that the equation for C alone would become

$$C = -r.R + g.G + b.B.$$

It will be noted, in particular, that only *three* matching stimuli are used to derive this equation.

If four radiations are mixed, a colour *C* can be matched with the same, but no greater, precision than with three, but there will now be many different combinations of the four stimuli that will yield a match. There will therefore be no unique equation for *C* in this case; further, the equation for *C* will still contain a negative quantity when *C* happens to be a spectral radiation.

These facts have been generally understood for the past 60 or 70 years, and were formulated in precise algebraical terms by Ives in 1915 and by Guild in 1924.

Essential amino-acid supplement and renal hypertrophy.

By C. REID. *Physiology Department, London Hospital Medical College*

A high-protein diet of lean meat only caused renal hypertrophy in rats and mice, but the addition of non-essential amino-acids alone, such as glycine and alanine, as dietary supplements to the ordinary standard diet of these animals was ineffective (Reid, 1944). Recently, more important amino-acids such as phenylalanine, tyrosine, arginine, lysine, tryptophane and methionine were available and were given mixed in the standard diet as single supplements in sufficient quantity to double the total N intake. These dietary supplements did not produce renal hypertrophy. Therefore renal hypertrophy depends on the intake of whole protein and not on selected supplements of single essential amino-acids.

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Protein sparing by carbohydrate with special reference to the hypertrophied kidney. By C. REID. *Physiology Department, London Hospital Medical College*

During a 24–48 hr. fast by rats and mice, carbohydrate given as cane sugar *ad lib.* decreases the breakdown of the protein of the liver, as judged by its total N content at the end of the fast, but does not affect that of the kidney or of the heart. A high-protein diet of lean meat only for 1–2 weeks increases the total N of the liver and kidney in rats and mice kept previously on the standard diet but does not increase that of the heart. As a preliminary investigation of the functional importance of the increased protein of the hypertrophied kidneys the protein sparing action of carbohydrate was tested as before. Results compared with those from controls showed that the protein of the liver is spared again but not that of the kidney or of the heart.

An attempt to determine some of the factors controlling the rate-of action of curare. By P. E. B. HOLMES, D. J. JENDEN and D. B. TAYLOR. *Departments of Physiology and Pharmacology, King's College, Strand, London*

Ing & Wright (1932) gave the value of the temperature coefficient Q_{10} of the paralysing action of tetramethylammonium iodide on frog sartorius as 1·5 and stated that the value suggested a chemical rather than a physical process.

An investigation of the effect of temperature on the action of curare has shown that the process, from a physico-chemical standpoint, is complex. If a slip of rat diaphragm (Bülbring, 1946) is paralysed with curare the rate of paralysis is decreased by a fall of temperature and this is in general consistent with a process limited either by diffusion or a chemical reaction, but when the temperature of a paralysed or partially paralysed muscle is lowered the paralysis can be reversed. This does not exclude diffusion as a rate-limiting process at high curare concentrations but invalidates the conclusion that the estimated overall Q_{10} of such a process is the temperature coefficient of a chemical reaction the velocity of which falls with temperature. If we regard the reaction between curare and the receptor as a reversible dissociation which increases with decreasing temperature we are forced to conclude that the reaction between curare and the receptor is endothermic. As a result of these considerations it is difficult to avoid the conclusion that there are at least two different factors contributing to any overall Q_{10} we attempt to measure.

The antagonistic effect of a fall of temperature on the action of curare has also been noted by Brown, Bülbring, and Burns and was mentioned by them during the discussion of their paper at the previous meeting of the Physiological Society.

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The transmitter substance of sensory nerve fibres. By H. HELLAUER and K. UMRATH. *Physiological Institute—Dir. Prof. H. F. Häusler—and Zoological Institute—Dir. Prof. v. Frisch—of the University of Graz*

Sensory nerves contain much less acetylcholine than motor and other cholinergic nerves (Loewi & Hellauer, 1939). To look for a transmitter substance in sensory fibres we extracted dorsal and ventral roots from freshly slaughtered cattle, boiling them for one minute in a solution containing 0·65 % NaCl and 0·01 % $CaCl_2$, 3 c.c. being used for 1 g. of nerve. Other methods of extraction seemed to be less favourable. As it is assumed to-day that an axon reflex in sensory nerve fibres of the skin produces a capillary dilatation, we tested our

extracts on the ear of the rabbit, injecting 0·1 or 0·2 c.c. into the convex surface subcutaneously. Generally extract D from the dorsal roots produced a spot of a deeper red than did extract V from the ventral roots, if compared 8-20 min. after injection. Eight minutes are necessary not only for extract D to become effective but also to do away with an early effect often produced by extract V. In many such cases the contrast became more pronounced when atropine was added to both extracts; and as the acetylcholine present in extract V is just about sufficient to redden the rabbit's ear, it may be the cause of this early action. Denervation of the ear much reduces the effect of histamine, acetylcholine and extract V, whereas that of extract D is not reduced but, often augmented. It now takes 15-40 min. for full development.

To purify the extracts we added conc. NaOH 1 : 50, evaporated the water, dissolved the residue in alcohol and transferred to chloroform. On the small intestine of the guinea-pig and of the cat the purified extracts were ineffective, while histamine was easily detectible in these tests in dilutions ineffective on the rabbit's ear.

Using the method employed by v. Muralt & Zemp (1943) for estimating vitamin B_1, we found in the original extracts V and D amounts of this substance corresponding approximately to that which they found in the unexcited frog's nerve.

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A slow infusion apparatus. By L. BERNSTEIN (introduced by C. REID). *Physiology Department, London Hospital Medical College*

The apparatus provides a means of infusing fluids into experimental animals at a slow and accurately controlled rate. This is achieved by the use of a modified hypodermic syringe with a threaded cap and a screwed rod replacing the usual cap and plunger-rod. If the barrel of the syringe is fixed, the revolutions of the screw drive the plunger forward in the barrel.

The screw can be driven by a clockwork or electric motor, but their high driving rate makes considerable gearing down necessary if the screw is to be driven at a suitable rate for very slow infusion. The use of ordinary clocks with their own reduction gearing is ruled out by the very limited power available.

The apparatus described uses a synchronous electric clock motor giving 1 r.p.m. at its seconds shaft. This carries an armature with two contacts from which a fixed wiper leads off two impulses a minute to operate a relay, which in turn operates the impulse motor of a standard G.P.O. selector switch. Fifty

pulses cause one complete revolution of the motor, which therefore makes one revolution in 25 min. The motor is coupled to the driving screw of the syringe by means of a keyed shaft and a flexible coupling, and the syringe delivers 1 c.c. in 50 min.

The clock is operated from the a.c. mains supply, and the impulse motor and relay require either 12 or 24 V., d.c., which can be obtained from batteries. The synchronous clock could be replaced by a spring driven one.

The rate of infusion can be altered by using different numbers of contacts on the clock armature so as to drive the impulse motor at different rates.

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The action of di-isopropyl fluorophosphonate, DFP, on the isolated rabbit heart preparation. (Preliminary communication.) By J. P. QUILLIAM and F. G. STRONG. *King's College, University of London*

The most active member of the alkyl fluorophosphonate group of compounds, di-isopropyl fluorophosphonate (DFP), has been shown by Adrian, Feldberg & Kilby (1942) to be a powerful miotic and lethal in small doses to animals. American workers have since confirmed that this compound is a very powerful anti-cholinesterase. It appeared probable that this substance would be a useful agent in experimental work where an eserine-like effect was required. The isolated rabbit's heart preparation was selected for a series of experiments designed to study the action of DFP.

The reactions of the heart to selected quantities of acetylcholine before and after DFP were studied. In the concentrations employed, viz. 0·25–2·5 mg., DFP markedly inhibited the heart. After a short period the heart regained its previous vigour. This temporary inhibition did not appear to be related to the anti-cholinesterase action of DFP.

After DFP the following changes were seen:

(*a*) There was a marked potentiation of the effect of acetylcholine in concentrations in which it previously had had an action.

(*b*) There was a sensitization of up to ten to one hundredfold if the duration of cessation of cardiac activity with subsequent recovery may be used as a measure for comparison.

(*c*) These changes occurred very rapidly after the administration of DFP by a single injection into the perfusion fluid and appeared permanent, being unaltered by washing.

The pharmacological effect of DFP upon the normal rabbit heart is thus similar to that of eserine but differs in that the changes occur very rapidly and seem to be permanent. DFP is a much quicker, simpler and surer method of sensitizing the rabbit heart to acetylcholine than is eserine. These results are in accord with those of Claydon & Quilliam (1947) in which the isolated frog rectus muscle preparation showed a permanent sensitization to and the frog heart a potentiation of acetylcholine after DFP.

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The influence of riboflavin on hydrochloric acid production in the human stomach. By H. LEHMANN, R. J. ROSSITER and J. H. WALTERS. (*India Command*)

As far as we are aware hitherto no defined chemical compound has been shown to have an activating influence when given to individuals whose stomachs respond subnormally to stimulus by test meals or histamine injections. We have found that riboflavin treatment improved HCl production and emptying time of the stomach in such cases. The subjects were either suffering from post dysenteric debility or from starvation syndrome. The following table summarizes results obtained in a series of ten repatriated Indian prisoners-of-war from the Far East suffering from starvation and stomach disfunction.

Test meal: 1 pint of oatmeal gruel.   Histamine: 0·75 mg. subcutaneously.

+ achlorhydria, histamine-resistant.
+ + achlorhydria, responding to histamine.
+ + + hypochlorhydria, less than 18 ml. N/10 free acid per 100 ml.
+ + + + normal free acid, emptying time above 2 hr.
+ + + + + normal curve.

Subject	Treatment	Dose in mg./day	Duration in days	Type of curve Before	After
1	Nicotinic acid	150 by mouth	3	+ +	+
1	Nicotinic acid	300 by mouth	7	+	+ +
1	Nicotinic acid	300 by mouth	14	+ +	+
2	Nicotinic acid	150 by mouth	3	+ + +	+ +
2	Nicotinic acid	300 by mouth	7	+ +	+ +
2	Nicotinic acid	300 by mouth	14	+ +	+ + +
4	Nicotinic acid	300 by mouth	7	+ + + +	+ + +
6	Nicotinic acid	300 by mouth	7	+	+
1	Riboflavin	4 parenterally	3	+	+ +
2	Riboflavin	4 parenterally	3	+ +	+ +
2	Riboflavin	4 parenterally 12 by mouth	6	+ + +	+ + + + +
3	Riboflavin	9 parenterally	14	+ +	+ + + + +
4	Riboflavin	4 parenterally 12 by mouth	7	+ + +	+ + + +
5	Riboflavin	12 parenterally	7	+ + + +	+ + + + +
6	Riboflavin	4 parenterally 12 by mouth	7	+	+ +
6	Riboflavin	4 parenterally	17	+ +	+ + +
7	Riboflavin	12 by mouth	7	+ + + +	+ + + + +
8	Riboflavin	4 parenterally 12 by mouth	7	+ + +	+ + + + +
9	Riboflavin	4 parenterally 12 by mouth	7	+ +	+ + +
10	Riboflavin	4 parenterally 12 by mouth	4	+ +	+ + +

Two theories of HCl production by stomach mucosa have been put forward lately. Both involve the oxidation of glucose in which riboflavin is a catalyst. The first (Conway & Brady, 1947) suggests an exchange of intracellular H+ —arising from the oxidation of glucose to organic acid—against K+ from

previously excreted KCl. The second (Davies, Longmuir & Crane, 1947) suggests the excretion of H^+ by electric force the energy for which is again provided by the oxidation of glucose.

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Respiratory rhythm and rhythm of body movement in the human.

By J. A. SAUNDERS. *Department of Physiology and Biochemistry, King's College, Newcastle upon Tyne*

An 'exercycle' was used to produce body movement of the sitting subject. The movement was upwards and forwards, the vertical and horizontal components each being $7\frac{1}{2}$ in. and the rate 37 times per min. The legs could remain stationary or be driven round in a pedalling movement 56 or 74 times per min.

Respiratory tracings showed that out of twenty-four subjects, ten breathed exactly in rhythm with the bucking movement, expiration starting during the downward movement of the body.

Two subjects investigated in more detail showed that:

(1) Respiration remained in rhythm with the bucking movement when passive leg movements (56 or 74 per min.) were added to the bucking movement.

(2) Voluntary pedalling with no load (56 per min.) while bucking gave a respiratory rate different from 37 per min. (usually greater).

(3) With the subject lying prone at right angles to the forward movement, respiratory rate remained at 37 per min. while bucking. It is deduced that the rhythm is probably not due to a piston action of the abdominal viscera on the diaphragm.

The thermal radiation inside the eye and the red end of the spectral sensitivity curve.

By M. H. PIRENNE.* *The Davy Faraday Research Laboratory of the Royal Institution, London*

If the dark-adapted retina were as sensitive to radiations of $\lambda = 1\text{-}1\cdot5\mu$. as it is to radiations of $0\cdot5\mu$., we should, in the complete absence of external light, see a continuous diffuse glow of light. For infra-red radiation of the above wavelengths is always present inside the eye because the eye is a 'black-body' radiator at blood temperature—in the same way as visible light is always present inside an oven at a temperature of, say, 1500° C.

Planck's formula gives the spectral distribution of the radiation in a 'black body'. At blood temperature the number of quanta $h\nu$ striking an area of 1 cm.^{2}/sec. is $5 \times 10^{-1} h\nu$ for the wave-lengths between 0·8 and 0·9μ.; $7 \times 10^2 h\nu$ between 0·9 and 1·0μ.; $2 \times 10^8 h\nu$ between 1·0 and 1·5μ.; $2 \times 10^{11} h\nu$ between 1·5 and 2·0μ. On the other hand, for $\lambda = 0.51\mu$., the minimum number of quanta which must strike the retina in order to stimulate it is of the order of 50 $h\nu$. This refers to a maximum retinal area of the order of 0·003 cm.^{2} and to a maximum duration of the order of 0·1 sec. It follows that a continuous flux of about $50/(0.003 \times 0.1) \approx 1.5 \times 10^5 h\nu$/cm.^{2}/sec. would produce stimulation over most of the retina. Now, while the black-body radiation is much smaller below 1·0μ., it is about 1000 times greater than this value between 1·0 and 1·5μ., and it would stimulate the retina if the latter had the same sensitivity to quanta of light of these wave-lengths as to those of $\lambda = 0.51\mu$.

If, therefore, the sensitivity curve did not drop sharply between 0·51 and 1·0μ., the infra-red radiation filling the eye would be seen and would interfere with night vision. Thus there is at the long-wave end of the spectrum a natural limit to visual sensitivity curves, and the actual curve of the human eye comes near to this limit.

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Secondary effects of insulin on metabolism. By C. REID. *Department of Physiology, London Hospital Medical College*

When mice fasted for 18 hr. are given by stomach tube 1 c.c. of one of the following solutions (18 % w/v), glucose, lactate, pyruvate, alanine, glycogen storage occurs in 3 hr. in the liver. On the other hand, glycogen storage is reduced by injecting insulin, 0·03 unit/20 g. mouse, when the glucose is fed, but the store of glycogen can be increased by giving more glucose, e.g. 1 c.c. of a 25 or 50 % solution. The mice given alanine, lactate or pyruvate show signs of hypoglycaemia before the end of the 3 hr. period and a negligible store of glycogen and appear to behave similarly to controls given insulin only. Increasing the quantity of alanine or lactate fed when insulin is given is ineffective in restoring liver glycogen, whereas glucose + alanine with insulin is as effective as glucose with insulin.

It appears, then, that one of the secondary results of giving insulin to fasting mice is to check deamination of amino-acids and the conversion of lactate and pyruvate, possible 3C atom derivatives from alanine, to glycogen. In addition, the relation of carbohydrate to protein metabolism is shown by the sparing action of carbohydrate but not of fat on the hepatic protein of normal but not of depancreatized rats.

Further the metabolic rate of normal mature cats, fasted 48 hr., is about 25 kg.cal./m.² body surface/hr. When insulin, 0·6–0·7 unit/kg., is given their blood sugar falls but not sufficiently to cause restlessness. Under these conditions the metabolic rate decreases slightly or insignificantly and the R.Q. remains at or near 0·72, the protein-fat level. Clearly, therefore, since the metabolic rate is not altered significantly by a dose of insulin of the above order, the rate of fat metabolism must also be unchanged.

Insulin hypoglycaemia in relation to diet. By C. REID. *Department of Physiology, London Hospital Medical College*

Mice fed on a high-protein diet of lean meat for 2 weeks and then fasted for 18 hr. develop signs of hypoglycaemia after an injection of insulin, 0·1 unit/20 g. mouse, more slowly than fasted controls previously kept on the standard laboratory diet or non-fasted mice on a high-fat diet. The delayed hypo-glycaemic response of the fasting protein-fed animals is due to their initial higher blood sugar.

TABLE 1. Blood-sugar fasting (18 hr.) mice fed previously on standard, high-protein, bread and high-fat diets

Standard diet		High-protein diet		Bread diet		High-fat diet not fasted	
Av. wt. (g.)	Blood sugar (mg. %)	Av. wt. (g.)	Blood sugar (mg. %)	Av. wt. (g.)	Blood sugar (mg. %)	Av. wt. (g.)	Blood sugar (mg. %)
18±2 (8)	91±10	18±3 (7)	110±12				
20±3 (8)	83±8	20±3 (8)	113±12				
Insulin 0·09 unit/20 g. mouse: blood sugar 75 min. later.							
19±1 (8)	41±5	19±1	58±6				
Insulin 0·1 unit/20 g. mouse: blood sugar 75 min. later.							
26±3 (5)	26±3	23±3 (6)	37±4	18±2 (4)	32±3	19±3 (11)	32±4

The figures after ± give the standard deviation of the mean.

Further, the protein-fed mice have more protein in their livers and excrete more nitrogen in the fasting period than controls. Presumably the greater fasting blood sugar depends on the higher rate of hepatic gluconeogenesis from protein.

Effect of *cis*-aconitic and *l*-isocitric acid on the synthesis of acetyl-choline. By N. EMMELIN (*Lund*) and W. FELDBERG.* *Physiological Laboratory, University of Cambridge*

Citrate has been found previously to have a strong accelerating effect on the enzymatic synthesis of acetylcholine (Nachmansohn, John & Waelsch, 1943; Nachmansohn & John, 1945; Feldberg & Mann, 1945–6). The action of citrate

is dependent on the presence of Mg ions (Feldberg & Hebb, 1947). Recently, through the kindness of Prof. H. A. Krebs, we had the opportunity of examining two organic acids which are related to citric acid and form part of the citric acid cycle: *cis*-aconitic acid and *l*-isocitric acid. Both these acids were found to have a strong activating effect on the *aerobic* synthesis of acetylcholine comparable with that of citric acid. Since these experiments have been performed a paper by Lipton & Barron (1946) appeared in which the replacement of citrate by *cis*-aconitate has been shown in the *anaerobic* synthesis of acetylcholine. Our results are illustrated by two experiments given in Table 1.

TABLE 1

μg. acetylcholine synthesized in 1 hr. at 37° C./g. acetone-dried brain

Molar concentration of acid added	Exp. 1		Exp. 2	
	Citrate	*cis*-Aconitate	Citrate	Isocitrate*
0	80	80	48	48
0·00015	540	340	300	250
0·0015	580	600	480	460
0·0037	—	—	—	420
0·0075	1150	1100	820	350

* The concentrations refer to the optically inactive synthetic compound, half of which is the physiologically active *l*-form.

Saline extracts of acetone dried rat's brain, after dialysis at 10 to 1° C. for 4 hr. (Exp. 1) and 4½ hr. (Exp. 2) respectively, were incubated at 37° C. for 1 hr. Each sample contained in 4·5 c.c. saline solution, buffered with sodium phosphate, the equivalent of 50 mg. acetone-dried brain powder, 3 mg. choline, 6 mg. KCl, 2 mg. NaF, 0·5 mg. eserine sulphate, 4 mg. MgCl₂, 4·5 mg. cysteine and 0·4 mg. ATP-P₇. The method has been described by Feldberg & Mann (1946). *Trans*-aconitic acid under similar conditions has no or a trivial effect only. It remains to be seen if the two acids, *cis*-aconitic and *l*-isocitric, act by themselves or through citric acid.

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The diameters and intercentre distances of the foveal cones. By H. HARTRIDGE. *From St Bart's Hospital Medical College, London, E.C.* 1

The diameters of the foveal cones, which have been measured by many workers, vary between 5·4 μ. (Kölliker) and nearly 1 μ. (Polyak, 1941). The evidence for the latter, which consists of three photomicrographs to the edges of which

scales have been attached, is contained in Fig. 40 of his book. Three methods have been used by the author of this communication for determining the diameters and intercentre distances of the cones in the photos: (1) to determine the diameters of selected cones, (2) to determine the number of cones in line in a given length, and (3) to count the number of cones in a given area.

The smallest cones were found, by method (1), to have diameters of not less than $1\cdot5\mu$., which, allowing for shrinkage ($\times 1\cdot31$, Osterberg, 1935), would be not less than 2μ. The average intercentre distances, allowing for shrinkage, were by method (2), left upper photo $4\cdot05\mu$., and right upper photo $4\cdot10\mu$.; and by method (3), left upper photo $4\cdot18\mu$., and right upper photo $3\cdot98\mu$. These give an average value of $4\cdot1\mu$.

It was possible to find small retinal areas in which the cone centres were less than $4\cdot1\mu$. apart; but no area was found, in the two upper photos, in which this distance was substantially less than 3μ. The lower photo in Fig. 40 of Polyak's book had poorer definition and was at a lower magnification than the two upper photos; measurements of the intercentre distances obtained from it were found to be unreliable.

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Electric rectification in frog's muscle. By B. KATZ and C. H. LOU.
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A subthreshold d.c. is sent through a sartorius muscle, and its transverse impedance is measured at anode and cathode, with an a.c. bridge. During the passage of the d.c., impedance changes are shown on an oscilloscope. In normal muscle, the impedance diminishes at the cathode and increases at the anode (Cole & Baker, 1941; Katz, 1942). This effect has been likened to the action of an electric rectifier element (Cole, 1941). If the potassium concentration on the outside of the muscle is raised more than three or four times, this apparent 'rectifier effect' reverses, the impedance now increasing at the cathode and diminishing at the anode.

The reversed effect is seen also by examining the electrotonic potential. With a potassium concentration of, for example, 120 m.equiv./l., the catelectronic potential rises in a prolonged fashion to a greater height than the anelectrotonic. The effect is clear with KCl, but becomes much more striking when Cl is replaced by a non-penetrating anion such as SO_4.

In *normal* muscle, one might expect the catelectrotonic potential to be smaller than the anelectrotonic, but the opposite is true. The reason for this discrepancy may be the occurrence of an active readjustment of the membrane

potential, similar to the local response in crustacean nerve described by Hodgkin & Rushton (1946).

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Transverse stimulation of muscle with alternating current. By B. KATZ and C. H. LOU. *Biophysics Research Unit, University College, London*

As was shown by Rushton (1927), a constant current applied transversely to nerve or muscle is ineffective because only a minute fraction of the current can penetrate the fibres and practically the whole flows through the surrounding fluid. An apparent paradox is obtained when a curarized sartorius muscle is exposed to an a.c. field, at frequencies above 200 cyc./sec. In this case, transverse current is more effective than longitudinal. The demonstration is facilitated by raising the potassium concentration 4–5 times, so as to prevent propagated impulses and restrict contraction to the parts which are directly stimulated. The whole muscle contracts in the transverse field (at, for example, 2000 cyc./sec.), only the ends contract in the longitudinal field (at, for example, 10 cyc./sec.). If only a portion of the muscle is traversed by a.c., at, for example, 2000 cyc./sec., this region can be shown to become depolarized and to contract when the depolarization exceeds a few mV.

To explain these effects, it may be recalled that the cathodal half-cycles, at each stimulated point, cause a local depolarization (Katz, 1939) which sums during successive cycles and reinforces the events at other stimulated points nearby. In transverse application of current, the effective stimulating electrodes are separated only by the fibre diameter and cover the whole length of the fibres.

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Contractility of muscle in isotonic potassium salt solution. By B. KATZ and C. H. LOU. *Biophysics Research Unit, University College, London*

Frog's sartorius muscle can be immersed for many hours in a potassium-rich solution (e.g. 120 m.mol./l.) without irreversible loss of its function, provided swelling of the fibres is prevented. This can be done, for example, by adding

potassium chloride to a solution containing sodium chloride or cane-sugar, or by using a non-penetrating anion such as sulphate (see Overton, 1904; Conway & Moore, 1945). Even while surrounded by an isotonic K_2SO_4 solution, the muscle does not completely lose its contractile power. A d.c. (0·5–0·7 mA.) is sent through the muscle, via non-polarizable electrodes, and maintained for several seconds. There is no visible response during the current flow, but at the break a local contraction is seen in the vicinity of the anode. The effect is similar to the anodal restoration of depolarized nerve described by Woronzow (1924) and Lorente de Nó (1944).

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Rapid equipment for recording muscle heat. By B. C. ABBOTT, A. C. DOWNING and A. V. HILL. *Biophysics Research Unit, University College, London*

At this meeting in 1939 two of us (with O. H. Schmitt) showed a recording galvanometer (Hill, 1938), with photoelectric coupling and thermionic amplification, for muscle heat measurement. Work with this was stopped by the war but has recently been resumed on rather different lines. The present equipment uses a galvanometer of about 5 msec. period, in a special anti-vibration carriage, electrostatically and acoustically shielded, and coupled by a twin-vacuum photo-tube and amplifiers to one beam of a double-beam cathode-ray oscillograph. The galvanometer is connected to a highly insulated thermopile about 50μ thick. For a single twitch the movements of the galvanometer (necessarily insensitive because of its speed) are magnified about 200 times on the screen; this implies a 20-millionfold amplification of the few microvolts from the thermopile. Following 'instantaneous' heating of the muscle on it, the thermopile gives half its full current in a few milliseconds and the galvanometer introduces little extra lag.

The second beam records any of the following: (*a*) the stimulus; (*b*) the isometric contraction (by a 'strain gauge' consisting of a single 60μ resistance wire); (*c*) the isotonic contraction (by similar or other means); or (*d*) the moments at which movement begins and ends, in after-loaded, released, or limited contractions.

Photographic records of a few sweeps starting shortly before the stimulus are taken in a fixed plate camera. They consist usually of a series of fine spots made by grid modulation at fixed intervals (for example of 2 or 10 msec.). They

are read in an enlarging camera with a photographic grating by which all errors due to distortion or non-linearity are automatically eliminated.

The chief result obtained hitherto is that the heat production in a twitch has already reached a high rate before the first sign of contraction is detected. The rapidity of the new equipment and the possibility of immediate correlation of thermal and mechanical events will facilitate a closer analysis of the processes associated with shortening, lengthening and the doing of mechanical work.

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Ionic exchange and fibre contraction. By J. L. MONGAR and
A. WASSERMANN. *Biophysics Research Unit, University College, London*

Opaque, slightly elastic and birefringent calcium alginate fibres were prepared which were capable of heterogeneous cation exchange reactions. On replacement of the calcium by alkali ions an initial contraction of the fibre followed by a lengthening took place. In a typical experiment a calcium alginate fibre, fully swollen with water, was hung over a hook and rinsed with 1 N sodium carbonate at a rate of flow of 0·7 c.c./g. fibre/sec. There was a contraction of the fibre of 13 % after 30 sec., when 29 % of the calcium in the fibre had been replaced by sodium (demonstration). During the contraction the weight, the transparency and the elasticity of the fibre increased while the birefringence decreased.

One possible explanation of these effects and of the results of similar experiments with a number of other salts (e.g. phosphates, oxalates, fluorides or citrates) is as follows. It is assumed that the bivalent calcium ions form salt bridges between adjacent alginate chains thereby building up a three-dimensional network in which the chains formed by repeating *d*-mannuronic acid residues must be relatively straight. On replacing some of the calcium by alkali ions, gel segments will be liberated which are more flexible than the completely 'vulcanized' structure. The segments will be able, therefore, to take up a statistically more probable configuration in which the chains are curled up to a certain extent, and this will lead to a shortening of the fibre as a whole. The contraction is regarded as a partial gel-sol transition, an interpretation which is also in concordance with the observed change of birefringence, transparency and elasticity.

It appears that the ionic exchange here considered gives rise also to some plastic flow; this simultaneous process is probably responsible for the final lengthening of the fibre and under certain experimental conditions it may even become predominant at an earlier stage.

PROCEEDINGS

OF THE

PHYSIOLOGICAL SOCIETY

17 *May* 1947

Use of the phosphatase reaction in a method of demonstrating bile capillaries in rats. By F. JACOBY. *Department of Physiology, The Medical School, Birmingham*

The presence of bile in bile capillaries of rats' liver can occasionally be revealed histochemically by means of the alkaline phosphatase reaction, though only in a very irregular and patchy manner (Jacoby, 1946). It was thought that by tying the bile duct and so causing a congestion of the liver with bile, involving also a raised alkaline phosphatase content, it should be possible to demonstrate the bile capillary bed more completely. Experiments of this nature were in a large measure successful. The animals were killed from 2 to 5 days after ligation of the bile duct, and small pieces of liver were fixed in alcohol, embedded in paraffin, sectioned and treated according to Gomori's method to show alkaline phosphatase. Such sections often show the bile capillaries over wide areas clearly and discretely outlined by black with narrow light lumina in between, whilst the control sections, incubated without the substrate (sodium β-glycerophosphate) are entirely negative. The nuclear structures of the liver cells are strongly positive, the cytoplasm is usually rather brown, and often the whole liver cell is outlined in black, also where it is adjacent to a sinus. The demonstration of the bile capillaries with this method appears equal if not superior to the old silver methods and has the additional advantage of being based on a functional and rational approach.

The well-known rise of alkaline phosphatase in the blood after bile duct ligation is histochemically reflected in a heavier and more extensive black staining of blood capillaries than normal, not only of those of the liver but also of those of other organs. This might indicate that in general the positive reaction (and its intensity) given by the endothelium of blood capillaries depends on the presence (and level) of the enzyme in the blood plasma, rather than on actual phosphatase activity of the endothelial cells themselves.

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PROCEEDINGS

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21 *June* 1947

A single sensory-ending preparation. By J. A. B. GRAY.
From The National Institute for Medical Research, London

Electrical records have been taken from single Pacinian corpuscles in the cat's mesentery. Some corpuscles occur in the transparent mesentery, away from the mesenteric arches, but in close association with fine vessels. With the mesentery stretched on a silver plate, the corpuscle was raised by a fine silk thread and the artery and vein running to it dissected as free as possible; the nerve has never been seen, but lies in association with the vessels. With 100μ platinum-wire electrodes (leading to cathode followers), applied to the corpuscle and its pedicle, records have been obtained of the action potentials both initiated in the corpuscle and produced by antidromic impulses in the mesenteric nerve.
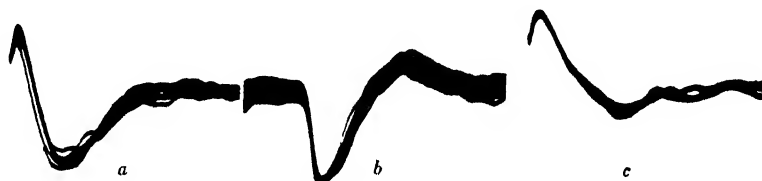

Fig. 1 *a*. Spontaneous action potentials triggering time base. *b*. Stimulated antidromic action potentials; stimulator triggering time base. *c*. As *b* but input reversed and action potential triggering time base. Time scale, 1 msec. = 22 mm.

In order that spontaneous potentials could be recorded with relatively high-sweep velocities, the time base was made so that it could be triggered by the first few volts of the amplified action potential. An electronic switch circuit in the triggering line enabled single transients or other samples to be photographed during a discharge from the corpuscle.

With one electrode on the corpuscle, slight vibration or even noise is sufficient to produce a discharge from the corpuscle. Injection into the corresponding mesenteric artery can give rapid access of drugs to the corpuscle. When the preparation is stimulated antidromically it behaves as one axon, although it is certain that there are normally other fibres in the pedicle; these, however, presumably conduct slowly and do not interfere with the main axonic potential.

'Spontaneous' and antidromic action potentials are shown in Fig. 1. The photographs are of many superimposed sweeps. From experiments already carried out it seems probable that the difference in shape of these potentials is caused by the presence of an active corpuscle, but physical explanations have not been finally excluded.

The action of isotonic potassium chloride solution on the electro-cardiogram of the frog. By W. F. FLOYD*. *Department of Physiology, Middlesex Hospital Medical School, London*

Hoff, Nahum & Kisch (1941) and Hoff, Nahum & Kaufman (1941) claim that the application, to the mammalian ventricular epicardium, of pledgets of filter-paper soaked in isotonic KCl solution, abolishes the electrical potential at the site of application. By this means they analysed the standard lead electro-cardiograms (e.c.g.) into components contributed by the right and left ventricles and by their anterior and posterior surfaces.
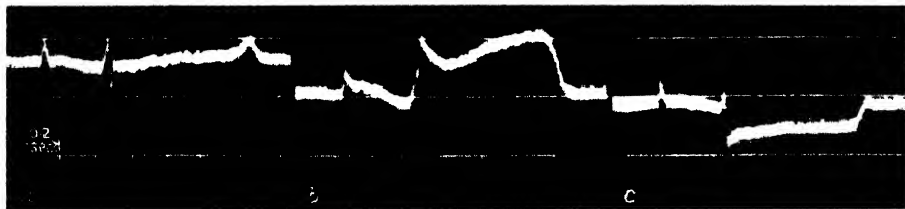

Fig. 1.

The normal e.c.g. of the frog as figured by Lewis (1925) resembles the mammalian e.c.g. in showing *P*, *R*, *S* and *T* waves (Fig. 1*a*). Isotonic KCl solution applied to the ventricle yields e.c.g.'s similar in appearance to the mammalian dextro-cardiogram (d.c.g.) and laevo-cardiogram (l.c.g.) obtained by Hoff *et al*. Lead I, with KCl applied to the left side of the frog's ventricle, yields a d.c.g., and vice versa (Figs. 1*b*, *c*).

The frog d.c.g. thus obtained is due to the surface electrical activity of a half-ventricle, and similarly for the frog l.c.g. Thus from an electrical point of view it would seem that one can regard each ventricle of the mammalian heart as a ventricular half-shell.

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The action of di*iso*propylfluorophosphonate on neuromuscular transmission. By G. L. BROWN, B. DELISLE BURNS and W. FELDBERG.* *National Institute for Medical Research, London, N.W. 3*

The action of di*iso*propylfluorophosphonate (DFP) has been examined on the tibialis anterior muscle of decerebrate cats. Administration of $100\,\mu g$. DFP by close arterial injection causes a potentiation of the twitch from maximal motor nerve volleys at 10 sec. intervals. The tension increases in the first few minutes after the injection and may attain three times the original twitch tension; thereafter the tension declines gradually, and, within the next 30–40 min., may be less than the original twitch tension. With larger doses—200–$1000\,\mu g$.—the onset of the potentiation is more rapid, but the tension of successive contractions rapidly declines, and, within a few minutes it may be much less than before. During the potentiation spontaneous twitches of the muscle occur. The increase in tension after DFP is due to repetitive contraction of the muscle; this repetitive discharge is still evident in electrical records even when the contraction has declined below the original twitch tension.

During and after the potentiation, stimulation of the nerve at 50–200/sec. for 5–10 sec. produces a poorly sustained contraction which is followed by a transient depression of the response to single nerve volleys. The quick contraction produced by arterial injection of acetylcholine ($2\cdot5\,\mu g$.) is not much changed by DFP, but it is followed by a profound and lasting depression of the response not only to nerve stimulation, but also to direct electrical excitation of the muscle.

The effects of DFP by arterial injection thus generally resemble those of eserine, given by the same route, and differ from them only in that eserine is effective in smaller doses and that depressant effects are much more evident after DFP. Given intravenously in doses up to 1 mg./kg. DFP has no visible effect on muscle tension or action potential, but appears to increase the effect of small doses of eserine given intravenously. Intravenous injection of large doses (10 mg./kg.) produces, after a latency of some 6 min., a small increase in the response to single volleys, and an interpolated tetanic stimulation is followed by a lasting depression.

Further experiments upon the effect of methionine on the nitrogen loss after burning. By M. DE G. GRIBBLE, R. A. PETERS and R. W. WAKELIN. *Department of Biochemistry, Oxford*

Results have been reported from this Laboratory (Croft & Peters, 1945) which show a decrease in the N loss in the urine after burns in rats by addition of 1 % methionine to the diet: on the other hand, Sellers & Best (1947) only obtained this upon diets previously deficient in methionine.

During the course of work planned with the object of throwing further light upon this problem we have had occasion to repeat these experiments, once in 1945 and three times in 1946, and have been unable to observe any effect of methionine upon the course of the N balance. It will be recalled that the earlier work showed that the N loss after burning amounted to 400–500 mg./rat for 9 days. Table 1 summarizes the results of the 1946 experiments, 2*a* giving an

TABLE 1. Mean differences in N excretion (urea + NH_3.N) in rats due to burning, expressed as mg./rat/day, and as total for 9 days after burning. All experiments were curative, the addition to the 10% casein diet being made after burning

Exp.	Addition	No. of rats Burned	No. of rats Unburned	Increase due to burning in mg. (urea + NH_3) N per day	9 days extra excretion
1	1% *dl*-meth. (95 mg.)	6	—	70·3 ± 16·66	632·7
2 (*a*)	*dl*-Alanine	6	6	40·0 ± 14·49	360·0
(*b*)	100 mg. *dl*-meth. 50 mg. *dl*-cysteine 10 mg. choline	6	6	37·4 ± 14·49	336·6
(*c*)	8% casein	6	6	28·8 ± 14·49	259·2
3 (*a*)	*dl*-Alanine + B vitamins	6	6	32 0 ± 7·00	288 0
(*b*)	1% *dl*-meth. + B vitamins	6	6	41·7 ± 7·00	375·3

Further statistical treatment of Exp. 2 'mean' excretions after burning adjusted for 'mean' excretions obtained before burning (Snedecor, 1946):

2 (*a*)	30·6 ± 5·71	275·4
(*b*)	58·6 ± 5·99	527·4
(*c*)	28·4 ± 5·64	255·6

improved statistical treatment. In these experiments, estimations have also been made of the urine passed in the feeding bowls, and there has been more efficient temperature control. Though the samples of methionine, wheat starch and yeast have been different in the later experiments, it has not been proved that these have caused the difference; the results suggest that the nutritional state before the experiment, or the intestinal flora may have been different in 1943–4, owing to wartime stock diets.

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